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Using centrifugal elutriation we have separated exponentially growing ML-1 cells into specific cell cycle populations and have treated these discrete fractions with Camptothecin for 6 hours. These experiments allowed us to determine that Camptothecin-induced apoptosis and activation of p53 via stabilization and p phosphorylation occur in all stages of the cell cycle. We have also determined that p53 mediated activation of the mdm2 gene occurs from a constitutively nucleosome free region. Additionally we have observed that differential activation of p53 induced genes does not occur at the level of chromatin reorganization. Finally we have observed that mutant p53 and Sp1 can be coordinately purified from an Sp1 DNA affinity column.

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TITLE: The In Vivo DNA Binding Properties of Wild-type and Mutant p53 Proteins in Mammary Cell Lines During the Course of Cell Cycle

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FOREWORD

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P. Bangor 7/31/98

PI - Signature Date

Table of Contents:

Introduction:

The purpose of this work has been to determine if different potential p53 DNA binding sites in nuclear chromatin are bound by different forms of wild-type p53 during the cell cycle. We are also working to determine if a direct DNA-mutant p53 protein interaction can occur iwhich may account for mt p53 gain of function (Dittmer et al. 1993). His 273 mutant p53 is able to activate HIV-LTR driven transcription and thus activate latent HIV replication. We are using this as our model system for the mutant p53 gain of function (Deb et al. 1994;Duan et al. 1994).

Most previous experiments examining the DNA binding properties of p53 proteins have been carried out *in vitro*, on naked DNA, with purified p53 protein produced either in bacteria or insect cells. Experiments with mammalian derived p53 proteins made use of predominantly crude cell extracts. During the course of this funded work we have studied the ability of wild-type and mutant p53 proteins to interact with DNA sequences (known to be bound by wild-type p53 in cell free systems) in nuclear chromatin. The studies presented in this final report were carried out with a number of different types of cells. Many of the cell lines are from breast tissue however to facilitate answering specific questions many different types of cell lines have been used. I

A revised statement of work for the grant proposal was accepted in August of 1996. This is the statement of work addressed in this progress report.

Background:

Biological functions of wild-type and mutant p53

The p53 gene encodes a protein which can function to suppress progression through the cell cycle in response to DNA damage (Ko and Prives 1996;Hartwell 1992;Lane 1992). It is present in minute concentrations in normal cells and tissues while being maintained at high levels in tumors and tumor cell lines due to post-translational stabilization (Oren et al. 1981). The p53 gene product is a phosphoprotein that is usually found in the nucleus although at times it is also found in the cytoplasm (Rotter et al. 1983) (Davis and Wynford-Thomas 1986;Gannon and Lane 1987). Mice deficient for p53 develop normally but are susceptible to tumor formation (Donehower et al. 1992). Studies on human tumors derived from colon, breast, brain, bone, and lung tissue, along with some studies on rodent cell transformation have shown that the wild-type p53 gene product functions as a suppressor of neoplastic growth while mutation or deletion of the wild-type gene can inactivate the gene products ability to carry out this suppression (Baker et al. 1990) and references within). Furthermore, several oncogenically transforming viruses have specific mechanisms to inactivate p53 function, strongly suggesting that inactivation of p53 is critical for efficient viral replication and efficient cell growth (Lane and Crawford 1979;Linzer and Levine 1979;Scheffner et al. 1990;Yew and Berk 1992). Mutations in p53 can cause cells to become oncogenically transformed (reviewed in (Yew and Berk 1992;Levine et al. 1991 & Zambetti and Levine 1993)) and mutant p53, in cooperation with ras, can transform cells. Consistent with its role as a tumor suppressor, wild-type p53 suppresses the oncogenicity of mutant p53 plus ras in focus forming assays (Eliyahu et al. 1989; Finlay et al. 1989). Quantitative improvements of transformation frequencies (Hinds et al. 1989; Dittmer et al. 1993) are associated with

the higher expression levels of mutant p53 protein, implying that such proteins altered amino acids impart a gain of function. It is interesting to note that the p53 gene has been conserved during evolution as demonstrated by the comparison of human, rat, mouse, frog, chicken, and bony fish cDNA clones which reveal five blocks of highly conserved sequence within the coding region (Soussi et al. 1990). Importantly, the point mutations found in several forms of human cancer (e.g. breast, lung, colon) occur predominantly in these conserved regions (Baker et al. 1990).

Wild-type p53 binds to DNA and regulates transcription

The p53 tumor suppressor protein can function as a checkpoint factor (Hartwell 1992) causing cells exposed to DNA damaging agents to either undergo growth arrest or programmed cell death (Hall et al. 1993;Kastan et al. 1991). Growth arrest mediated by p53 can occur at both the G1/S and a G2/M borders. The normal function of p53 is regulated, at least in part, by the ability of the protein to bind site-specifically to DNA (Vogelstein and Kinzler 1992). The tumor-derived mutant p53 proteins that have been tested thus far have altered, or deficient DNA binding activity (Bargonetti et al. 1991;Chen et al. 1993;Kern et al. 1991;Kern et al. 1991), however some oncogenic p53 mutants can bind to DNA at temperatures lower than 37°C (Bargonetti et al. 1993;Zhang et al. 1994) or to idealized p53 binding sites (Hupp et al. 1993;Zhang et al. 1993). Wild-type p53 binds nonspecifically to DNA (Kern et al. 1991; Steinmeyer and Deppert 1988) as well as specifically to diverse DNA sequences that contain two adjacent copies of the consensus sequence 5'-Pu Pu Pu C (A/T) (T/A) G Py Py Py-3' (el-Deiry et al. 1992;Funk et al. 1992). It has been predicted that between 200 and 300 of these p53 response elements (REs) exist in the human genome (Tokino et al. 1994).

In response to DNA damage nuclear p53 levels rise (Kastan et al. 1991). p53 induced growth arrest or apoptosis can be brought about in part by specific activation of genes containing p53 REs. Some of the responsive genes in which p53 binding sites have been identified include the oncogene mouse double minute 2 (mdm2) (Barak et al. 1993;Juven et al. 1993;Perry et al. 1993), growth arrest and DNA damage 45 (gadd45) (Hollander et al. 1993;Kastan et al. 1992) (Zhan et al. 1993), p21/waf1/cip1 (el-Deiry et al. 1993), bax (Miyashita and Reed 1995) and 14-3-3 (Hermenking et al. 1997). The product of the p21/waf1/cip gene is a potent cyclin dependent kinase inhibitor. This presumably accounts for the ability of wild-type p53 to arrest cell cycle progression at the G1/S border before activation of the cyclin regulated p34 cdk2 kinase (el-Deiry et al. 1993;Harper et al. 1993). Additionally, activation of gadd45 (which is part of the DNA damage response pathway) may inhibit DNA replication until DNA repair has taken place (Kearsey et al. 1995;Smith et al. 1994). The p53 mediated growth arrest at the G2/M border appears to be through activation of a 14-3-3 gene which inhibits the activity of cdc25 (Hermenking et al. 1997). Activation of the bax gene is involved in the apoptotic signaling pathway (Miyashita et al. 1994;Miyashita and Reed 1995). The activation of mdm2 by p53 adds another level of complexity to p53 mediated activation due to the fact that the MDM2 protein binds to p53 and blocks p53 mediated transactivation (Chen et al. 1994). MDM2 can also promote p53 degradation (Haupt et al. 1997) (Kubbutat et al. 1997). Thus when Mdm2 is over-expressed it functions as an oncogene (Oliner et al. 1993). Over-expression of mdm2 in cancer cells is achieved by different mechanisms which include increased transcription (Sheikh et al. 1993), enhanced translation (Landers et al.

1994;He et al. 1994), gene amplification (Oliner et al. 1992;Refenberger et al. 1993) and gene rearrangements (Leach et al. 1993).

Differential occupancy of consensus binding sites in chromatin

Recent evidence has made it quite clear that all p53 responsive genes are not turned on under the same set of conditions. For example it has been shown that p53 mediated transcriptional activation of the mdm2 gene can be turned off while both p21/Waf1 and Bax are being activated (Thomas and White 1998). During the period of this funded project we have been working to determine if differential activation of genes by p53 is controlled at the level of the chromatin configuration. The local structure of inducible genes falls into two categories: preset and remodeling (Thomas and White 1998). Preset genes are those in which the binding sites for *trans*-acting factors are accessible as demonstrated by a DNaseI hypersensitive state. Additionally no major subsequent alterations in the chromatin structure of the promoter regionoccurs during gene activation. Remodeling genes are those genes in which some of the required *cis*-acting regulatory elements are packaged into nucleosomes.

While some transcription factors, like GAL4 (Taylor et al. 1991) and Sp1 (Li et al. 1994), can bind specific DNA sites within a nucleosome core, others like NF1 and heat shock factor, are unable to interact with specific DNA binding elements when the nucleosome core is assembled (Archer et al. 1991;Pina et al. 1990). In fact it is a presupposition that in general, nucleosomes positioned over promoters are inhibitory to other proteins binding, however it has been demonstrated that this is not always the case (Reviewed in (Workman and Buchman 1993)). Therefore, it has been suggested that many genes are programmed during DNA replication while the nuclear chromatin assembles (Workman et al. 1990). If there are limiting transcription factors available in a cell then a gene that is replicated early in S-phase has more opportunity to assemble an active transcription complex than a gene that replicates late. This is because a gene that replicates early may be available for transcription factors to bind before all the early replicating portion of the genome has sequestered these factors. Additionally, transcriptional activators can stimulate eukaryotic DNA replication by modifying the outcome of the competition between initiator factors and histones for occupancy of the replication origin (Cheng et al. 1992).

Wild-type p53 is present in low levels in normal cells (Oren et al. 1981) and the level and activity of p53 increases when cells undergo DNA damage (Kastan et al. 1991). Because p53 needs to rapidly initiate a response it may be important for p53 to belong to the class of transcription factors that activates preset genes. However different p53 responsive genes can produce products that elicit different biological effects. Therefore some genes activated by p53 may be preset while others may be remodeling. In some cases p53 may be required to organize nucleosome structures in order to help define the p53 DNA binding sites that become active enhancer elements (McPherson et al. 1993). With this possibility in mind one could postulate that the p53 protein might bind to some specific sites during S-phase. Because the wild-type p53 protein has a short half life it may have to bind immediately in the presence of nucleosomes and therefore may only bind to sites that have nucleosome cores positioned in a particular way. It is also possible that p53 binding sites exist in different chromatin states over the course of the cell cycle (as is the case for yeast replication origins which bind various transcription factors(Diffley

et al. 1994; Dowell et al. 1994)). Dependent on the sequence context, p53 may function differently. Recent experiments by the Manfredi group suggest that his is the case (Resnick-Silverman et. al. 1998). Additionally, if p53 is a member of the class of activators which is blocked by the presence of nucleosomes, perhaps oncogenic mutation of the protein (and/or complex formation with other proteins) may confer, on p53, the ability to bind sites that the protein normally finds inaccessible. Mutations in p53 might change the spectrum of growth-control genes that the protein activates. Interestingly the RGC site is positioned near an origin of replication (Kern et al. 1991). Finally, mutant p53 can localize to the nucleus of cells and has been observed to interact directly with nuclear matrix attachment region (MAR) sequences (Muller et al. 1996). MARs are able to organize cellular chromatin into topologically independent loops (reviewed in (Boulikas 1995)). Therefore it has been proposed by Muller, Paulsen and Deppert that MAR-mutant p53 interactions may be involved in the mutant p53 gain of function (Muller et al. 1996).

Year Four with a DOD Career Development Award:

Revised Statement of Work Accepted 1996

The in vivo DNA Binding Properties of Wild-type and Mutant p53 Proteins During the Course of Cell Cycle

New Task 1:

Set up a new laboratory. Months 1-12.

a. Order equipment and reagents to supply an empty laboratory.
b. Recruit and train four students.
c. Begin tissue culture facility operation and characterizing breast cancer cell lines growth conditions.
d. Set up DNaseI sensitivity and *in vivo* footprinting conditions.
e. Purification of p53 from baculovirus infected insect cells.

New Task 2 = revision of old task 1:

Establish stable breast cancer cell lines expressing temperature sensitive p53 (ts-p53val135) while working with a murine established ts-p53val135 line. Months 7-36:

a. Examine p53 level and sub-cellular localization in the established murine cell line at both the p53 permissive and restrictive temperatures. Test ts p53 effects by Northern Blotting.

b. Attempts will be made to establish ts-p53val135 expressing breast cancer cell lines of MDA-MB 468, ZR 75, MDA-MB 157, MDA-MB 361 and MCF10A.

c. Levels of p53 in the established breast cell lines will be examined both at the p53 permissive and restrictive temperatures. Test ts p53 effects by transient transfection.

New Task 3 = revised task 2:

Footprinting of the MDM-2 and GADD45 p53 DNA binding sites in nuclear chromatin of unelutriated cells and on naked DNA. Months 12-36:

a. The p53 responsive regions of the MDM-2 and GADD45 genes will be obtained from laboratories that have published the clones.

b. Gel shift analysis of p53 binding elements with nuclear and cytoplasmic extracts.

c. Southern blot using the using the murine ts p53val135 cell line to set up the system and test chromatin structure of the gene.

d. Footprinting of mdm-2 and gadd45 binding sequences in chromatin and with immunopurified p53.

e. In vivo footprinting of the MDM-2 and GADD45 p53 binding regions will be carried out in breast cell lines with and without ts-p53val135, and also on the cell lines treated with chemotherapeutic agents.

New Task 4 = revised old task 3:

***In vivo* DNA footprinting of synchronous populations of hematopoietic and breast derived cell lines. Months 12-24.**

a. Synchronous populations of the various hematopoietic and breast cell lines will be prepared by both centrifugal elutriation and drug treatment.

b. Intranuclear footprinting on the synchronous populations of cells will be carried out.

New Task 5: This task addresses a similar to question to that of the old task 4, however we will focus on one potential binding site for mutant p53 rather than searching for many. Months 20-48.

Test to see if mutant p53 His273 is able to bind to the HIV-LTR region when it is transiently transfected into the cell line MDA-MB-468.

a. Examine HIV-LTR driven transcription in breast cell lines containing different status p53 protein.

b. Footprinting of the HIV-LTR region in the nuclei of breast cell lines containing different status p53 protein in both the absence and presence of chemotherapeutic drug treatment.

c. Comparison of the proteins from different status p53 breast cell extract bound to the HIV-LTR region.

Task 6 = old task 5:

Studies on the affect of p53 on the DNA replication of the double minute chromosomes in the breast cancer cell line MDA-MB 361. Months 12-48.

a. Examine the level of MDM-2 gene amplification level in MDA-MB 361 cell lines that express ts-p53val135, both at the permissive and restrictive temperatures.

b. Make and analyze MDA-MB 361 cell fusions with normal breast cells.

c. Carry out in vivo footprinting on synchronous populations of the above cell types.

Materials and Methods:

Isolation of Protein from mammalian cell culture lines:

Whole cell lysates: Wash 100 mM plate 2X with ice cold PBS
Extraction of cells on a 150 mm dish will be lysed with 2 ml of whole cell lysis buffer: (O.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% sodium dodecyl sulfate, 50 mM NaCl, 25 mM Tris.HCl (pH 7.5), 1 mM PMSF, 50 ug/ml aprotinin, 50 uM leupeptin) leave for 15 min. on ice. Scrape cells and put into tube. Lysates should be spun at 15,000 rpm for 15 min., and the supernatant saved.

Nuclear Extract Preparation:

Adherent cells nuclear extract was prepared as follows. Wash cells 2X with cold PBS. Add 2 ml of Lysis Buffer per Plate. Spin 2300 rpm for 5 min. - (Save the supernatant for cytoplasmic extract). Resuspend the pellet in 1.5 ml of nuclear extraction buffer. Transfer to an eppendorf tube and rock at 4°C for 60 min. Spin 10 min. in microfuge in cold room. **Lysis buffer stock:** 20 mM Hepes, pH 7.5, 20% Glycerol, 10 mM NaCl, 1.5 mM Mg Cl_2, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 1 mM PMSF, 50 ug/ml aprotinin, 50 uM leupeptin. **Lysis Buffer:** 8.8 ml Stock and 6.2 ml water. **Extraction Buffer:** 1.47 ml 5M NaCl, 8.8 ml of Stock, and 4.7 ml of water.
The procedure for the suspension ML-1 cells was adapted from (Kastan et al. 1992). The nuclei were lysed in 3 ml of nuclear lysis buffer for 1.0×10^9 and passed for 3 strokes through a 25 gauge needle.

Immunoprecipitation from cell extracts and Western Blot Analysis:

Normalize for total protein in the cell extract as indicated. Protein concentrations were determined using the Biorad assay reagent. Add 30-40 ul of 50% beads coupled to PAb421 to the extract. Rock 2-20 hours at 4°C in the cold room. Spin 5 minutes in the cold room. Wash beads 4X with 1 ml of RIPA buffer (150 mM $NaCl_2$, 50 mM Tris pH 7.2, 1% NP 40, 2% Na deoxycholate and 0.1% SDS). The beads were resuspended in 1X protein sample buffer and boiled. Samples were electrophoresed on a 15% SDS-PAGE and electrotransferred to nitrocellulose. The blot was probed a mixture of p53 monoclonal antibodies PAb240, PAb1801 and PAb421 and the signal was visualized after incubation with goat anti-mouse second antibody by developing with ECL solutions (Amersham).

Electroporation of Breast Cell Lines:

MDA-MB-157 and MDA-MB-468 cells are grown in DMEM with 10% fetal calf serum. ZR75-1 cells are grown in RPMI 1640 with 10% fetal calf serum. Cells were washed in medium without serum and harvested with trypsin. Electroporation at .23kV was carried out with $0.5 \times 10^{(6)}$ cells/pulse in medium without serum. The amount of DNA was varied from 1 to 20 ug and selection for the different cell lines was carried out at the lowest G418 concentration determined to kill all the cells of a control un-electroporated plate.

Electrophoretic Mobility Shift Assay (EMSA):

Nuclear extracts were prepared as described above. EMSA was carried out using 0.1 pmoles of radio-labeled oligonucleotide (either a p53 super consensus sequence (SCS) binding site oligonucleotide, the DNA binding site from the gadd45 gene (gadd45), the Sp1 binding sites form the HIV-LTR (HIV) or a mutated p53 binding site oligonucleotide (Mut) and 2 ug of nuclear extract from either MDA-MB-468 or ZR75-1 cell lines maintained at 37°C. The DNA binding reactions were carried out at room temperature for 20 minutes in a 30µl volume containing 20 mM Hepes(pH 7.8), 100 mM KCl, 1 mM EDTA, 1 mM DTT, 1 µg of sheered salmon sperm DNA and 10% glycerol in the presence of PAb 421 antibody.

Extraction of Nuclei and DNaseI Treatment and *in vivo* Footprinting:

This protocol was adapted from (McPherson et al. 1993)Cells were grown on 150 mM plates to no more than 80% confluence before shifting the temperature using 10 plates per analysis sample. Wash plates with ice cold PBS 2X. Spin down at low speed at 4°C (2500 rpm in SS34 rotor) for 10 min. Resuspend in 2 ml of RSB with PMSF. Homogenize 20 strokes and check for trypan blue exclusion. Spin down at 4000 rpm 4°C, 10 min. Wash nuclei in 2 ml RSB with out PMSF 1X. Resuspend in any where from 1 ml to 4 ml of RSB w/o PMSF (make the lowest # of nuclei in 1 ml for 4 tubes at 250 ul per tube the rest to accordingly making sure to normalize for number of cells i.e.. estimate number of cells from confluence and count in hemocytometer). Set up reactions for DNaseI treatment. **RSB:** 10 mM Tris pH 7.4, 10 mM NaCl, 3 mM MgCl2 pH 7.4, 0.5% NP40, 1 mM PMSF or absent. Treat with DNaseI for 10 min. at 37°C, using 250 ul of nuclei in 0.1 mM $CaCl_2$. Add 250 ul DNaseI stop (with out DNA) and then add proteinase K to a final concentration of 400 ug/ml and digest overnight at 37°C. Extract once with phenol and 3X with chloroform. **DNaseI stop:** 2M NH4 OAc, 100 mM EDTA, 0.2% SDS. **Southern Blot Analysis** was carried out using the standard technique described in Protocols of Molecular Biology. **Ligation Mediated PCR** was carried on the DNA samples as described by Mueller and Wold (Mueller and Wold 1989)

Centrifugal Elutriation of ML-1 and MANCA cells:

Centrifugal elutriation was carried as previously described for the MANCA cell line (Koff et al. 1992;Soos et al. 1996). Elutriation of ML-1 cells has not been published, but the conditions were similar to those used for MANCA cells with the exception that the cells took a little longer to pump out because they appear to be larger.

Results:

This DOD Career Development Award was instrumental in helping the lab become established. We have made a great deal of progress which has been described in the progress reports submitted oreviously. For this final report the progress over the last year will be stressed. For the sake of clarity and report length progress and problems that have been described in the past years progress reports will be omitted. We havebeen working to elucidate the chromatin structures of both the mdm2 and gadd45 genes (some of this data has been published (Xiao et al. 1998). We have set up a system where we can induce differential regulation of p53 inducible genes. We have also begun to study the regulation of p53 during the cell cycle by examining the biochemical functions of p53 in cell cycle fractions separated by centrifugal elutriation. Finally we have succeeded in co-ordinate purification of p53 with Sp1 from an Sp1 DNA affinity column.

Task #2: Establish stable breast cancer lines expressing temperature sensitive p53 (ts-p53val135) while working with a murine established ts-p53val135 line.

Task #2 a. Examine p53 level and sub-cellular localization in the established murine cell line at both the p53 permissive and restrictive temperatures. Test p53 effects by Northern Blotting.

Completed as described in the 1996 progress report.

Task #2 b. Attempts will be made to establish ts-p53val135 expressing breast cancer cell lines of MDA-MB 468, ZR 75, MDA-MB 157, MDA-MB 361 and MCF10A.

Completed as described in the 1997 progress report.

Task #2 c: Levels of p53 in the established cell lines will be examined both at the p53 permissive and restrictive temperatures.

Completed as described in the 1997 progress report.

Task #3 Footprinting of the MDM-2 and GADD45 p53 DNA binding sites in nuclear chromatin of unelutriated cells and on naked DNA.

Completed as described in the 1997 progress report. We carried *in vivo* footprinting on the nurine mdm2 gene and have shown that no chromatin changes occur at this p53 RE upon p53 DNA binding (Xiao et al. 1998). We are presently carrying out the gadd45 footprints. *In vivo* footprints of p53 binding to the gadd45 gene have been published recently, however the change in protection in the presence and absence of p53 was unimpressive (Chin et al. 1997). We believe that the reason only slight protection was observed in this study was due, in part, to the fact that exponentially growing cells were

used. We expect that we will achieve better results when we examine the gadd45 protection patterns in cell cycle fractions.

Task #3b: Gel shift analysis of p53 binding elements with nuclear and cytoplasmic extracts.

We have generated data which make it extremely clear that DNA damage elicits upstream signals that differentially affect the specific genes which p53 is able to transcriptionally activate. We have used the inducible p53 human cell line TR9-7 (Agarwal et al. 1995) in order to examine the affect of high levels of p53 in the presence and absence of DNA damage (Figure 1). Using Northern blot analysis we have seen that the mdm2 and p21 genes are activated in the presence of high levels of p53 in the absence of Camptothecin induced DNA damage, while under these same conditions very little activation of the gadd45 gene occurs (Fig. 1A, lane 4). In contrast, the gadd45 gene is activated in the presence of DNA damage induced by Camptothecin while the mdm2 gene is turned off and p21 mRNA production shows no change (Fig. 1A, lane 5). These differences do not appear to be controlled at the level of p53 DNA binding activity when monitored by EMSA (Fig. 1B, compare lanes 4 & 7) or by the p53 protein level when exteact are monitored by SDS-PAGE (Fig.1C, compare lanes 4 & 5) . These data suggest that perhaps post-translational modification of p53 or genomic chromatin configuration may influence aspects of p53 that are important for the way the protein regulates different promoters. The fact that the p53 induced in the TR9-7 cell line in the absence of tetracycline could only be supershifted by PAb1801 antibody and not the 421 antibody, while the p53 activated by Camptothecin induced DNA damage showed some reactivity to the p53 antibody PAb421 (Fig. 1B, compare lanes 6 & 9) points to a post-translational controlling event..

Task #3 C. Southern blot using the murine ts p53-Val135 cell line to set up the DNase I sensitivity assay and test chromatin structure of the gene.

Completed as described in the 1997 progress report.

Our determination that p53 binds to a constitutively nucleosome free region of the mdm2 gene (Xiao et al. 1998; paper attached) made it clear to us that at least one of the p53 responsive genes is preset. We believe that DNAseI sensitivity studies of other p53 binding sites is important and should be used as a tool to determine if p53 responsive genes are generally all preset. Drosophila heat shock genes are all preset (Wallrath et al. 1994). We have used indirect Southern blotting to assess the DNaseI sensitivity of the gadd45 gene in the breast cancer cell lines, the normal breast cell line MCF12A and the TR9-7 cell line described above. The nuclei from the various cell lines (grown with and without Camptothecin) have been extracted and treated with DNaseI. Agarose gel electrophoresis has been used to screen the samples for increasing DNaseI digestion (data not shown). The total DNA was then digested with EcoRI which is a viable enzyme for indirect Southern Blot screening of both the mdm2 gene and the gadd45 gene. The DNaseI curves were successful and the TR9-7 and MCF12A Southern blots have been probed for DNAseI sensitivity of the gadd45 gene (Figs. 2 & 3). In the TR9-7 and the MCF12A cell lines both the gadd45 promoter (Figs. 2 & 3, indicated by the arrow at 3kb)

and the gadd45 p53 RE (Figs. 2 & 3, indicated by the arrow at 1.3kb) show some DNAse hypersensitivity in the absence of activated p53. No change in DNAseI sensitivity was evident in the TR9-7 cells upon action of p53 by the withdrawal of tetracycline and the addition of Camptothecin (Fig. 2, TR9-7 -Tet&CPT), although gadd45 transcription was activated under these circumstances (Fig. 1A, compare lanes 3 & 5). Interestingly, in the MCF12A cell line an increase in DNAseI sensitivity at the gadd45 promoter was observed when the cells were treated with Camptothecin (Fig. 3, indicated by the arrow at 1.3 kb). These data suggest that the gadd45 gene is preset but in the presence of DNA damage in certain cell lines increased DNaseI sensitivity can occur at the gadd45 promoter region. We are in the process of examining the DNaseI sensitivity of the gadd45 gene in the breast cancer cell lines MDA-MB-468, MDA-MB-157 and ZR75-1. We will examine the DNaseI sensitivity of the mdm2 gene in these human cell lines using the mouse mdm2 indirect probe (which we have determined works) because we do not have a human genomic mdm2 clone.

Figure 1:

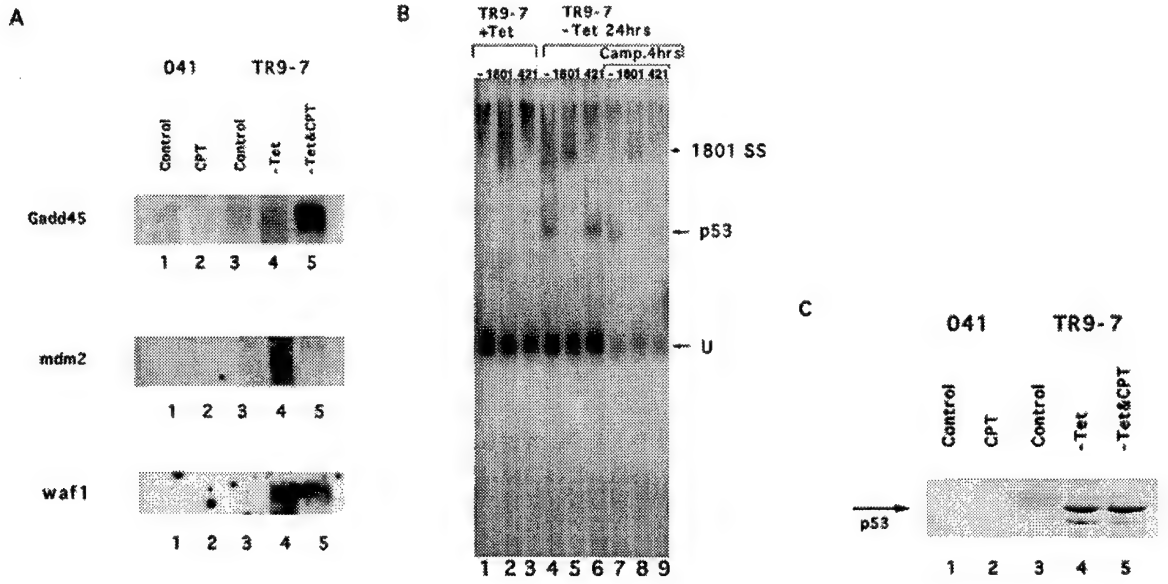


Figure 1: Differential activation of wt-p53 target genes does not correspond to p53 DNA binding activity and the protein level of p53. *The RNA extraction and nuclear protein extracts were made from cells grown in media containing tetracycline (+Tet), after 24 hrs withdrawal of tetracycline (-Tet), or after 24 hrs withdrawal of tetracycline with overlapping 0.1mM Camptothecin treatment for the last 4hrs. (A) Northern blot analysis of the wt p53 responsive genes was carried out by separating 0.5 ug of GAPDH normalized poly-A RNA in 1% formaldehyde-agarose gel and transferring to a positively charged membrane. The blot was sequentially hybridized with full length cDNA probes for Gadd45, mdm2, and waf1 as indicated. (B) The DNA binding reactions were carried out at room temperature for 20 minutes in 30 uL total volume in a mixture containing 0.15 pmoles of super consensus sequence (SCS) oligonucleotide, 2 ug nuclear protein extracts, 20 mM HEPES(pH 7.8), 100 mM KCl, 1 mM EDTA, 1mM DTT, 1 ug sonicated salmon sperm DNA and 10% glycerol in the absence (lanes; 1, 4 and 7) or presence of p53 antibodies 1801 (lanes; 2, 5 and 8) or 421 (lane; 3, 6 and 9). 10 uL of the reaction mixture was resolved on a 4% acrylamide gel at 200V at 4°C. (C) 150 ug of nuclear protein was resolved by electrophoresis on a 10% SDS-PAGE. The p53 in samples was visualized by Western blotting with a mixture of p53 specific monoclonal antibodies PAb 240 and PAb 1801 and detection with ECL reagent (Amesham).*

Figure 2:

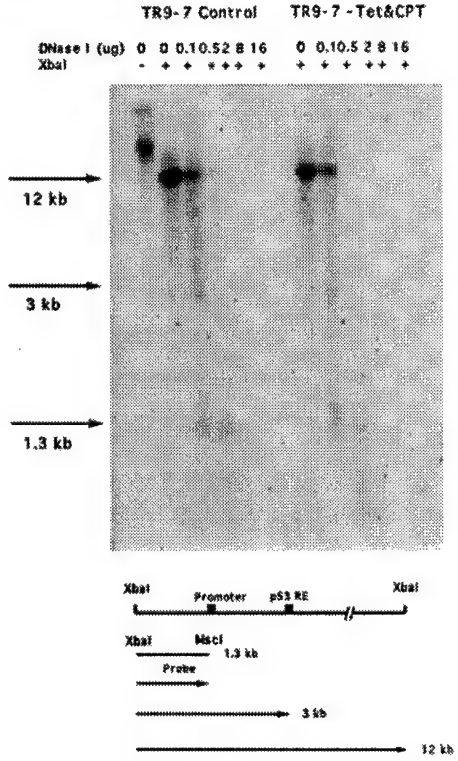
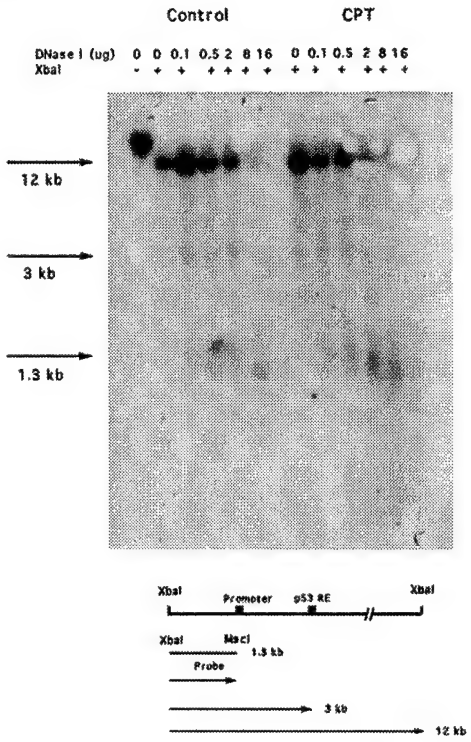


Figure 3:



Figures 2 & 3: The human gadd45 gene locus has a similar DNase I hypersensitive pattern to the murine mdm2 gene: i.e. Preset. *DNA in 2 x 10^6 isolated nuclei was digested with increasing amounts of DNase I (0 ug, 0.1 ug, 0.5 ug, 2 ug, 8 ug or 16 ug as indicated above each lane).*

(2) Nuclei were isolated from TR9-7 cells containing tetracycline (control) or after 24 hrs withdrawal of tetracycline with overlapping 0.1mM camptothecin treatment for the last 4hrs (-Tet&CPT).
(3) Nuclei were isolated from 184A1 cells with (CPT) or without (Control) camptothecin for 4 hours. Purified DNA was restricted with XbaI and electrophoresed on 0.8 % agarose gel, probed with a [^{32}P] labeled XbaI-MscI genomic gadd45 probe fragment.

Task 3 D. Footprinting of mdm-2 and gadd45 binding sequences in chromatin and footprinting with immunopurified p53.

Simultaneous intra-nuclear protection of the mdm-2 p53-REs and the TATA box

Studies on the ability of p53 to bind to DNA have been carried out *in vitro*, but little is known about the ability of the protein to bind to specific target sites *in vivo*. In order to elucidate how p53 activates initiation of transcription we focused on the DNA binding of the p53 to chromatin and thus carried out intra-nuclear ligation mediated PCR (LM-PCR) DNase I protection analysis. We began this analysis with one p53 responsive gene, mdm2 and have described the studies in past progress reports. The attached paper describes a large portion of the work (Xiao et al. 1998).

In order to begin ligation mediated footprinting of the gadd45 gene the primers had to be ordered and tested for there predicted melting temperatures. This design was described in the 1996 progress report. The primers were ordered and have proven to work successfully. They have enabled us to sequence the plasmid containing the gadd45 gene as well as determine the length of time needed for the sequencing gel run. The DNaseI treatment of the nuclei has been carried out as described above. The next step is to carry out LM-PCR on the successfully DNaseI treated chromatin.

Task #3e: Footprinting of the mdm2 and gadd45 sites in nuclear chromatin will be carried out in breast cell lines with and without ts p53-Val135, and also in cell lines treated with chemotherapeutic agents.

Breast cancer cell lines that express ts p53-Val135 have been selected, as shown in the 1997 progress report. DNase I sensitivity curves have been carried out in the breast cancer cell lines MDA-MB-468, MDA-MB-157 and ZR75-1 as well as in the stable clones. These samples are presently being used for both indirect Southern blot analysis as well as for the *in vivo* footprinting experiments. The data is forthcoming.

Task #4 : *In vivo* DNA footprinting of synchronous populations of hematopoietic and breast derived cell lines.

Task #4a. Synchronous populations of the various hematopoietic and breast cell lines will be prepared by both centrifugal elutriation and drug treatment.

Cell cycle analysis using elutriated fractions:

Centrifugal elutriation of tissue culture cells allows cell cycle factions to be separated on the basis of their migration in a gradient. This method has an advantage over using drugs to block the cell cycle at specific stages because drug treatment induces a number of responses and needs time to produce the various populations of cells. In the case of p53 DNA interaction research, by the time the cells are arrested it is possible to have missed the critical moment of the interaction. We are carrying out our elutriation experiments using the suspension cell line ML-1 (a myeloid leukemia cell line). The ML-1 cell line contains wild-type p53 that can be activated in response to DNA damage. Elevation in the p53 level in ML-1 cells is detectable in less than 1 hour after exposure to gamma irradiation (Kastan et al. 1991) and within 2 hours after treatment with the drugs bleomycin, actinomycin D, etoposide or Camptothecin (Nelson and Kastan 1994). Although the wild-type p53 status of the ML-1 cell line has been well documented no published data for a successful elutriation of this cell line exists. We have succeeded in setting up elutriation conditions for this cell line in order to carry out biochemical experiments investigating the DNA binding activity of p53 present during different stages of the cell cycle before and after the induction of DNA damage (this was show in the 1997 progress report).

We used centrifugal elutriation to separate ML-1 cell cycle fractions and then carried out an in depth study on the cells before and after Camptothecin induced DNA damage (Figures 4 and 5). We separated the ML-1 cells into cell cycle fractions by centrifugal elutriation. Half of each fraction was then treated with 20uM Camptothecin for 6 hours. For this elutriation experiment we analyzed the cell cycle distribution of the cells before and after drug treatment using FACS (Figure 4). Interestingly the late S and G2/M fractions continued moving through the cell cycle during the 6 hour Camptothecin treatment while the G1 and early S fractions did not. All the Camptothecin drug treated fractions appeared to exhibit approximately 40% to 55% sub-G1 DNA content suggesting that the amount of apoptosis that occurred during each cell cycle stage was approximately the same (data not shown).

We then proceeded to examine the p53 level in each fraction by Western blot analysis using different p53 specific antibodies (Figure 5a and 5b, non-elutriated and cell cycle fractions are as indicated). Using this method we observed that after drug treatment the level of p53 was increased in all the cell cycle fractions. The forms of p53 present after drug treatment slightly differed in the variable cell cycle fractions (Figures 5a and 5b: right half). Before drug treatment the amount of p53 was barely detectable (Figures 5a and 5b: left half). We also examined phosphorylation of p53 at serine 389 using a p53 phosphospecific antibody which was a generous gift from Dr. Gigi Lozano (Figure 5c). Dr. Lozano and colleagues have published that p53 is phosphorylated at serine 389 in response to UV induced DNA damage (Kapoor and Lozano 1998). We have noted that Camptothecin induced DNA damage can activate p53 phoshporylation at this residue however we did not observe cell cycle regulation of phosphorylation at this site (Fig. 5c). We would like to point out that p53 in ML-1 cells has recently been documented to form different cleavage products in response to DNA damage (Molinari et al. 1996;Okorokov et al. 1997). We have observed different size forms of the protein resulting after the cells are treated with Camptothecin. In addition to analyzing p53 we examined the amount of p21(the cyclin dependent kinase inhibitor that is activated by p53) in each cell cycle fraction using anti-p21 antibody (Oncogene Science). The results were surprising at first because although we did see a specific band appear after drug treatment it was smaller than we had expected. After a search of the current literature we noted that cleavage products of p21 have recently been reported (Poon and Hunter 1998). These p21 cleavage products are no longer able to inhibit cell cycle progression and apoptosis in the way that their full length counterparts do (Gervais et al. 1998;Lu et al. 1998). Therefore due to the fact that Camptothecin induces ML-1 cells to undergo apoptosis it is not surprising that the p21 present is not full length. Figure 5e demonstrates that the gel was normalized for total protein by demonstrating normalization for actin levels. Although DNA damage induced by the chemotherapeutic drug Camptothecin induces ML-1 cell apoptosis, the gamma mimetic drug Zeocin induces primarily ML-1 cell cycle growth arrest (Figure 6). We are very interested in understanding how p53 mediated growth arrest is controlled. In the coming year we will carry out the above experiments with the DNA damaging drug Zeocin.

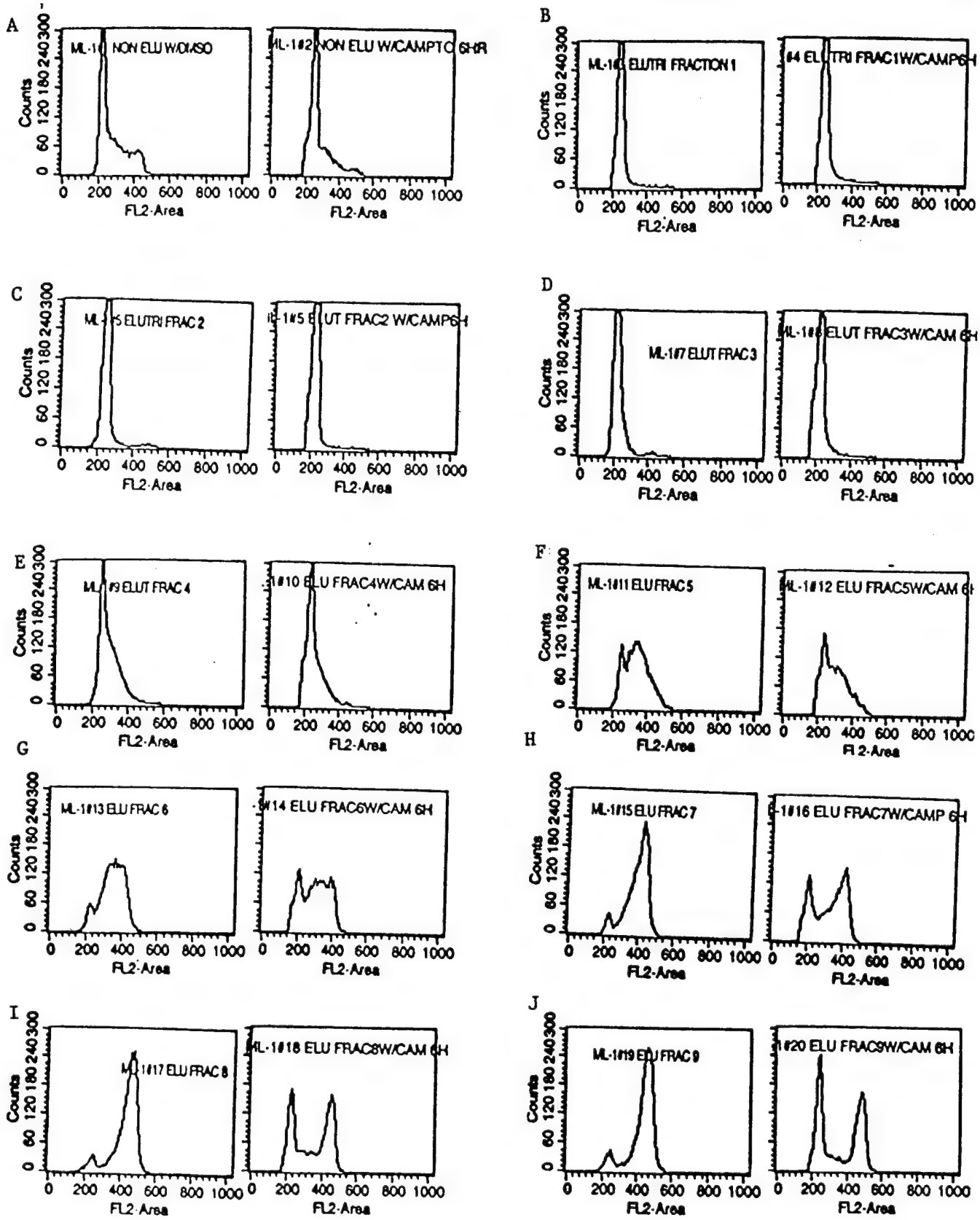


Figure 4: ML-1 cells separated into cell cycle fractions by centrifugal elutriation. Each fraction was then treated with 20um Camptothecin for 6 hours. All fractions are shown in pairs. Panel A: Non-elutriated ML-1 cells without and with treatment. Panels B-J: Cell cycle fractions without and with treatment.

Figure 5:

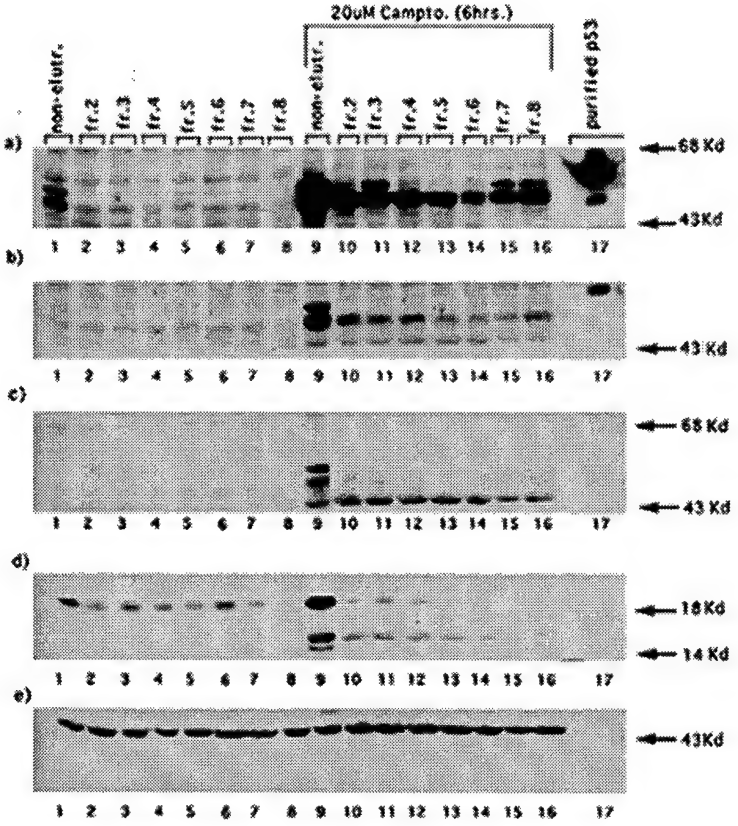
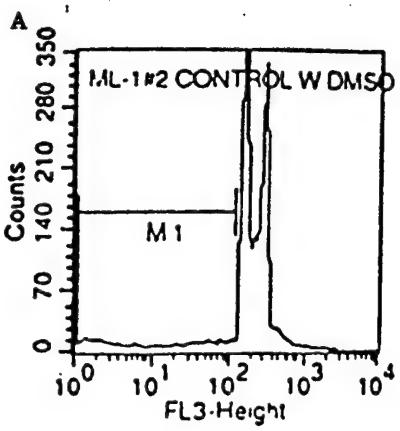


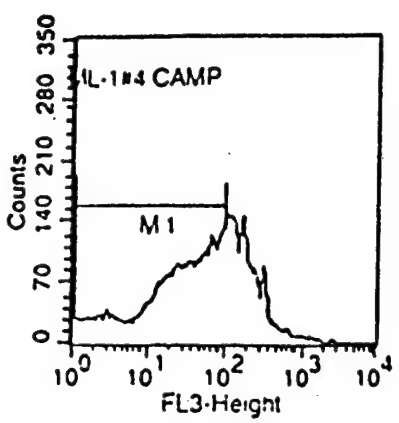
Figure 5: *Nuclear lysates from elutriated cell cycle fractions with and without camptothecin treatment. Protein-containing extracts (nuclear lysates) were prepared from elutriated and non-elutriated ML-1 cells grown in 1x RPMI with 10% fetal bovine serum. Exponentially growing cells were treated with 20uM camptothecin for 6 hours at 37'C. Extracts containing 95ug of nuclear protein were electrophoresed on a 10% polyacrylamide gel, transferred to a nitrocellulose membrane and analyzed for a) p53 protein levels by immunoblotting with anti-p53 antibody PAb 421 (specific to C-terminus) and PAb 1801 (specific to N-terminus), or b) PAb 240 (specific to central domain) alone, c) p53 specific phosphorylation at serine 389 with anti-p53 phosphoserine 389, and d) p21 protein expression with anti-p21 antibody. e) Blot was probed with anti-actin to normalize for loading. Arrows indicate the positions of molecular weight markers.*

In order to determine if differential p53 DNA binding activity exists during the various cell cycle stages we have examined the p53 DNA binding present in the nuclear extracts derived from the elutriation fractions. EMSA's were carried out using synthetic oligonucleotides derived from the p53-binding site of the gadd45 gene (Kastan et al. 1992) (Fig. 7a) as well as an oligonucleotide containing the DNA sequence determined to act as a super consensus site (SCS) (Halazonetis et al. 1993) (Fig. 7b). p53 binding was barely detectable in the absence of the p53 specific antibody PAb 421, therefore the binding analyzed was that induced by the addition of 421 antibody (Hupp et al. 1992). Nuclear extract from each cell cycle fraction (10ug) was incubated with [^{32}P]-dCTP labeled gadd45 or SCS oligonucleotide and then analyzed by electrophoresis on a 4% polyacrylamide gel (Fig. 7). As seen in Fig. 5 the level of p53 in the untreated samples

A



Marker	Left.	Right	Events	% Gated
All	1.	9910	24174	100.00
M1	1.	118	3482	14.40



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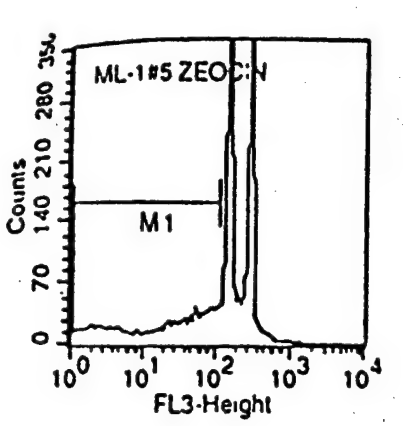
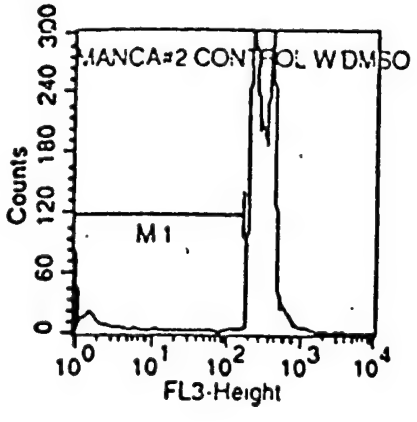
All	1.	9910	37653	100.00
M1	1.	96	25594	67.97



Marker	Left.	Right	Events	% Gated
All	1.	9910	26488	100.00
M1	1.	113	8616	32.53

B



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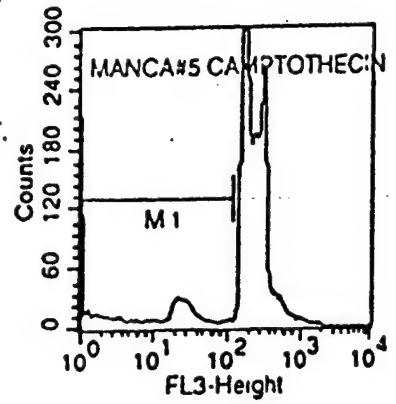
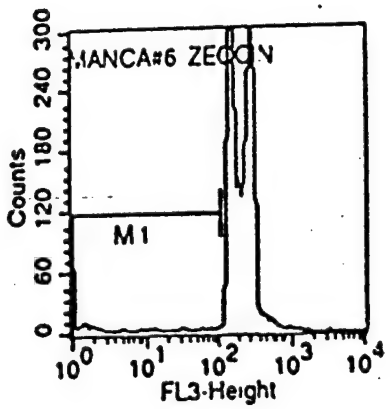
All	1.	9910	23335	100 00
M1	1.	164	1159	4.97



Marker	Left.	Right	Events	% Gated
All	1.	9910	26029	100.00
M1	1.	113	3336	12.82



Marker	Left.	Right	Events	% Gated
All	1.	9910	21534	100.00
M1	1.	108	680	3.16

Figure 6: FACs Analysis of ML-1 and Manca cells treated with camptothecin and zeocin. Panel A: ML-1 cells with no treatment, ML-1 cells treated with 20um camptothecin for 24 hours, ML-1 cells treated with 50ug/ml zeocin for 24 hours. Panel B: Manca cells with no treatment, Manca cells treated with 20um camptothecin for 24 hours, Manca cells treated with 50um zeocin for 24 hours.

was barely detectable and the p53 level in all the drug treated fractions showed a marked increase. The EMSA data was in agreement with the Western blot analysis showing a clear increase in the PAb 421 induced p53 gel shift in all the drug treated samples (Fig. 7, compare lanes 1-8 to lanes 9-16). Interestingly, although drug treated fractions 2 and 3 contained approximately the same amount of p53 more DNA binding activity was evident in fraction 3 both with the gadd45 and the SCS oligonucleotide (Fig. 7, compare lanes 10 and 11). Additionally the DNA binding activity observed in drug treated fractions 7 and 8 reproducibly varied between the gadd45 and SCS oligonucleotides, with fraction 8 showing more DNA binding activity for the SCS oligonucleotide and fraction 7 demonstrating more DNA binding activity for the gadd45 oligonucleotide (compare lanes 15 and 16). Increased and altered DNA binding activity has been demonstrated when p53 is phosphorylated *in vitro* with S and G2/M but not G1 cyclin-dependent kinases (Wang and Prives 1995). This suggested to us that we would see the highest p53 DNA binding activity in fractions 7 and 8 (which contained high percentages of G2/M cells). We compared the G1 fractions 2 and 3 to the G2/M fractions 7 and 8 because they contained approximately the same level of p53.. Overall fractions 7 and 8 had more DNA binding activity than fraction 2 but the DNA binding activity of fraction 3 was similar to that of fractions 7 and 8. These data suggest that the control of p53 DNA binding activity is not simply at the level of phosphorylation by different cyclin-dependent kinases.

Figure 7:

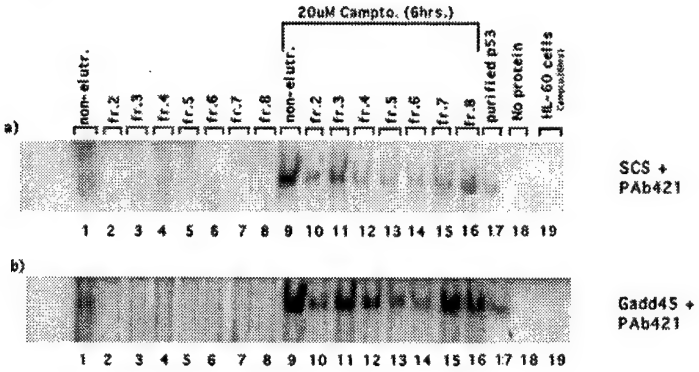


Figure 7: Mobility shift assays with Gadd45 p53 binding site & p53 super consensus site and extracts from camptothecin treated ML-1 cells. *Nuclear extracts from non-elutriated and elutriated ML-1 cells isolated after 6 hrs. treatment with 20uM camptothecin were incubated with [P32]-labeled DNA corresponding to the a) p53 super-consensus site (SCS) and b) the Gadd45 p53 binding site and then electrophoresed on a 4% acrylamide gel. HL-60 cells treated with camptothecin for 6 hrs. and samples containing no nuclear protein were used as negative controls. Wild-type p53 was used as a positive control.*

Task 4b. Intranuclear footprinting on the synchronous populations of cells will be carried out.

Camptothecin treatment of ML-1 cells induces cellular apoptosis while Zeocin treatment of ML-1 cells induces growth arrest (Fig. 6). We believe that in *vivo* footprinting of the gadd45 site should be compared in non drug treated and Zeocin drug treated fractions. Apoptosis adds another level of complexity to the problem that we would prefer to address after we have examined footprints under growth arrested conditions. We have

previously been able to see a clear *in vivo* footprint at the mdm2 p53 REs when cells have become growth arrested by p53. The experiments described for Camptothecin treated ML-1 cells will be repeated with Zeocin treated ML-1 cells and the chromatin footprints will be analyzed.

Task #5 Test to see if mutant p53 His273 is able to bind to the HIV-LTR region when it is transiently transfected into the cell line MDA-MB-468.

Task 5a. Examine HIV-LTR driven transcription in breast cell lines containing different status p53 protein.

The ability of mutant p53 His 273 to transactivate promoters containing Sp1 binding sites has been confirmed in our laboratory by introducing an HIV-LTR luciferase reporter construct into the MDA 468 cells. We have results indicating that HIV-LTR directed transcription is mutant p53 dependent since co-expressing a temperature sensitive (ts) p53 in a wild type conformation negates this effect (Figure 8, also shown in the 1997 progress report). This result indicates that the wt protein has a dominant effect over the mutant endogenous protein.

Figure 8:

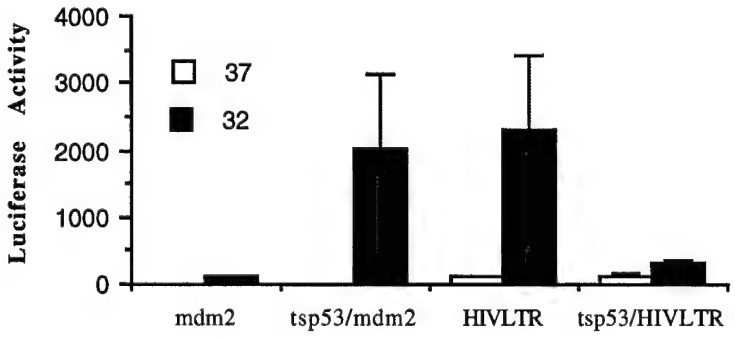


Figure 8: MDA-MB-468 transactivation of the HIV-LTR is inhibited by expressing a temperature sensitive p53 in its wild type conformation. *1 x 10^7 cells were transfected by electroporation at 350V with either 10μg of reporter plasmid (HIV-LTR or mdm2) and 10μg of carrier DNA or 10μg of reporter plasmid and 10μg of a plasmid encoding the ts p53 Val135 where indicated. At 48 hours post-transfection, cells extracts were made from cells maintained at 37°C and cells switched to 32°C for 24 hours. Data presented is the average value of duplicates. The mdm2 reporter is under regulation of the p53 binding site located in the mdm2 gene. HIV-LTR reporter is under regulation of the long terminal repeat of the HIV-1 (which has three Sp1 binding sites).*

When the grant proposal was first written we had proposed to use breast cancer cell lines that would stabily express ts p53 Val135 as a way to inactivate mutant p53 in a temperature controlled manner. These experiments have proved problematic in that the data from the MDA-MB-468 clone which stability expresses ts p53 Val135 (named 8-9 and shown in the 1997 progress report) does not agree with the data in Fig. 8. These data are presented in Fig. 9.

Figure 9:

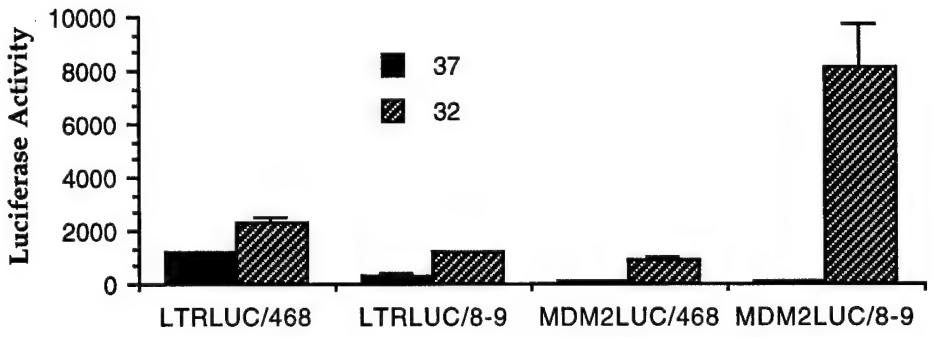


Figure 9: Transactivation of the HIV-LTR is inhibited in the 8-9 cell line expressing a temperature sensitive p53 in its wild type conformation. *1 x 10^7 cells were transfected by electroporation at 350V with 10 μg of the indicated reporter plasmid (HIV-LTR or mdm2), 2 μg of a plasmid expressing b-galactosidase and 8 μg of carrier DNA. At 48 hours post-transfection, cells extracts were made from cells maintained at 37°C and cells switched to 32°C for 24 hours. Data presented is the average value of duplicates. The mdm2 reporter is under regulation of the p53 binding site located in the mdm2 gene. HIV-LTR reporter is under regulation of the long terminal repeat of the HIV-1 which has three Sp1 binding sites. 8-9 is a clone of MDA 468 breast cancer cell line stably expressing the ts p53 Val 135*

Task 5b. Footprinting of the HIV-LTR region in the nuclei of breast cell lines containing different status p53 protein in both the absence and presence of chemotherapeutic drug treatment.

We have continued to struggle with the transient transfection experiments at the expense of carrying out in *vivo* footprinting on the HIV-LTR. The experimental design needs to be organized in a way that we can control the delivery of mutant p53 into the system. The transient co-transfection shown in Fig. 8 suggests that the best possible mechanism for examining this is by co-transfection of a mutant p53 expressing plasmid and the HIV-LTR reporter into a cell line containing no endogenous p53. We have decided to use the cell line 041 (which we have used before as in Fig. 1) for these co-transfection experiments. This work is in progress.

Task 5c. Comparison of the proteins from different status p53 breast cell extract bound to the HIV-LTR region.

A number of studies have discussed the protein-protein interaction of p53 and Sp1 (Bargonetti et al. 1997;Borellini and Glazer 1993;MacLeod 1993;Gualberto and Baldwin Jr. 1995;Ohlsson et al. 1998). We have chosen DNA affinity chromatography as a method to purify p53-DNA associated proteins in order to examine if mutant p53 and Sp1 can coordinately interact with DNA. In order to begin our analysis of DNA co-associated proteins we have prepared DNA affinity columns using the DNA-sepharose method of Kadonaga (Kadonaga 1991). An HIV-LTR and an SCS column were prepared using synthetic oligonucleotides from Operon. The HIV oligonucleotide contained three Sp1 binding sites and had the following sequence:
5'-GGATCCGGGACTTTCCAGGGAGGCGTGGCCTGGGCGGGACTGGGGAGTGGCGA-3'

The SCS synthetic oligonucleotide used in this study contained the following sequence:
5' TCG AGC CGG GCA TGT CCG GGC ATG TCC GGG CAT GTC - 3'

We have tested these columns for their ability to isolate mutant p53 and Sp1 from MDA-MB-468 nuclear extract. We found, using Western blot analysis of the HIV-LTR column fractions, that mutant p53 His273 co-eluted with Sp1 at 0.4M KCl (Fig. 10, lane 0.4). We further confirmed that p53 elutes in the 0.4M KCl fraction by EMSA of PAb 421 dependent p53 super-shift of an SCS oligonucleotide (Fig. 11, lane 12). These results show that mutant p53 from the breast cancer cell line MDA-MB-468 can associate with Sp1 binding sites co-ordinately with Sp1. We propose that this occurs in the MDA-MB-468 nucleus and may have some biological significance.

Figure 10:

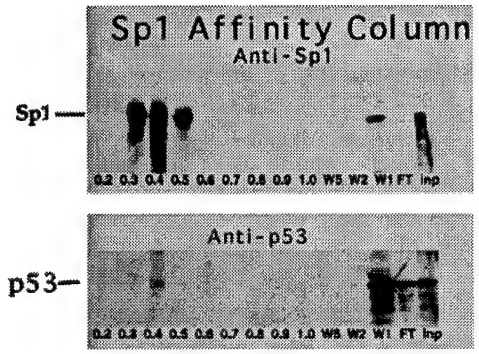


Figure 11:

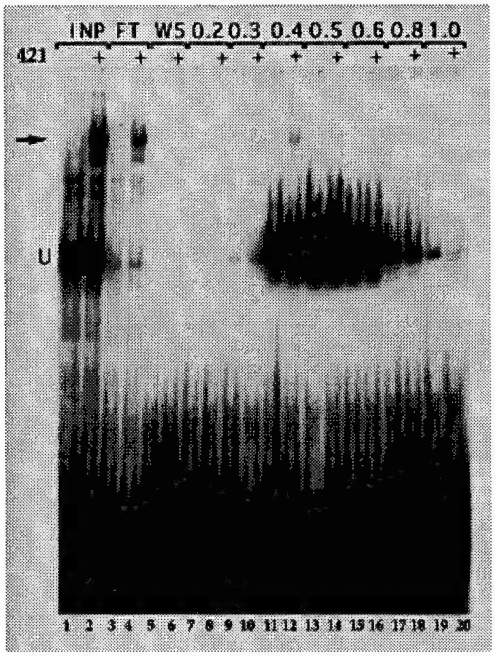


Figure 10: Mutant p53 His 273 and Sp1 from MDA MB 468 cells co-elute from a DNA affinity column containing Sp1 binding sites. *Nuclear extracts from MDA MB 468 cells were fractionated on a sephacryl S-300 column to eliminate nucleases. The fractions were combined, concentrated and 750 ug of protein was passed through a Sp1 DNA affinity column. The column was washed 5 times with buffer Z containing 0.1M KCl and the bound factors were eluted with increasing concentrations of KCl (0.2-1.0M). 1 ml fractions were collected and concentrated to 0.1 ml. 50 ul of each elution fraction was separated by SDS PAGE and transfered to a nitrocellulose membrane. The presence of p53 or Sp1 was determined by immunoblotting. W1, W2 and W5 indicated washes one, two and five respectively; FT indicates flow through and 'inp' indicates input (50 ug).*

Figure 11: Mutant p53 elutes from a Sp1 DNA affinity column at 0.4M KCl as determined by the p53 antibody 421 induce supershift on an SCS oligo. *Nuclear extracts from MDA MB 468 cells were fractionated on a sephacryl S-300 column to eliminate nucleases. The fractions were combined, concentrated and 1500 ug of protein was passed through a Sp1 DNA affinity column. The column was washed 5 times with buffer Z containing 0.1 M KCl and the bound factors were eluted with increasing concentrations of KCl (0.2M to1.0M). Each of the 1 ml fractions collected for the washes and elutions were concentrated to 0.1 ml. 3 ul of each fraction was used in the EMSA assay. The DNA binding reaction reactions were carried out at room temperature for 20 minutes in a 30 ul reaction volume in a buffer containing 0.1 pmoles of oligonucleotide, 3 ul of the indicated fractions, 20 mM Hepes(pH 7.8), 100 mM KCl, 1mM EDTA, 1mM DTT, 1 ug of sonicated salmon sperm DNA*

and 10% glycerol in the presence of a p53 antibodies (421) where indicated. 15 ul of each reaction mixture was resolved by electrophoresis on a 4% acrylamide gel at 200V at 4°C. INP indicates input (0.13%). FT is the flow through (0.13%) and W5 corresponds to wash number five (3 ul). The arrow indicates the p53 Ab 421 induced supershift and the "u" is a band corresponding to a unidentified binding factor.

The reciprocal experiment using the SCS affinity column was also carried out anddemonstrated co-elution of Sp1 and p53 in the 0.3M and 0.4M KCl fractions (Figures 12, 13 & 14). Western blot analysis showed predominant co-elution of Sp1 and p53 in the 0.3M KCl fraction (Fig. 12, lane 0.3), however EMSAs with both the SCS oligonucleotide (Fig. 13) and the HIV-Sp1 oligonucleotide (Fig. 14) showed p53 and Sp1 DNA binding activity in both the 0.3M and the 0.4M KCl fractions (Figs. 13 & 14, lanes 10 & 12). Although purified mutant p53 His 273 demonstrated slight DNA binding activity for the SCS oligonucleotide it did not demonstrate the ability to bind to the HIV-Sp1 oligonucleotide (Fig. 15, SCS -vs- HIV lanes 7-10). This suggests that the ability to see mutant p53 co-elute with Sp1 from the HIV column is not due to a direct interaction of mutant p53 with the Sp1 binding sites on the DNA. Additionally, we have been unable to co-immunoprecipitate mutant p53 His 273 and Sp1 from MDA-MB-468 nuclear extract (data not shown) and therefore suggest that another factor is responsible for the co-ordinate elution. It is possible that the band identified as "u" in the elution profile examined by EMSAs contains this protein factor. Experiments are in progress to determine the protein responsible for the "u" shift.

Figure 12:

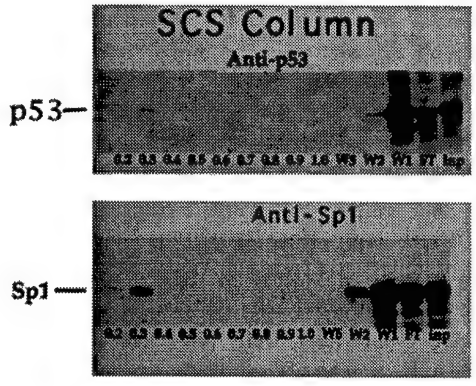


Figure 13:

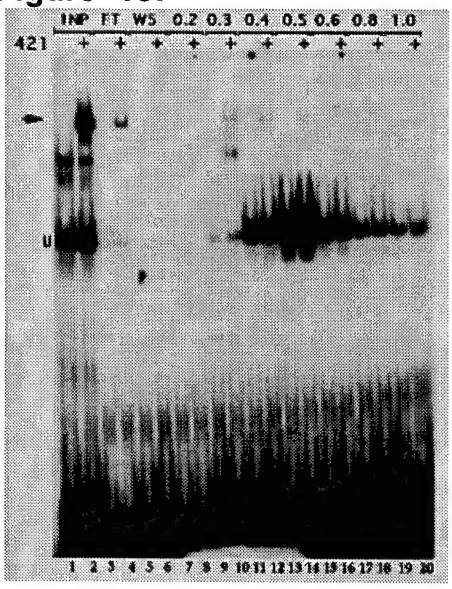


Figure 12: Mutant p53 and Sp1 from MDA MB 468 cells co-elute from a DNA affinity column containing a p53 super consensus binding site (SCS). *Nuclear extracts from MDA MB 468 cells were fractionated on a sephacryl S-300 column to eliminate nucleases. The fractions were combined, concentrated and 750 ug of protein was passed through a SCS oligonucleotide column. The column was washed 5 times with buffer Z containing 0.1M KCl and the bound factors were eluted with increasing concentrations of KCl (0.2-1.0M). 1 ml fractions were collected and concentrated to 0.1 ml. 50 ul of each elution fraction was separated by SDS PAGE and transfered to a nitrocellulose membrane. The*

presence of p53 or Sp1 was determined by immunoblotting. W1, W2 and W5 indicated washes one, two and five respectively; FT indicates flow through and 'inp' indicates input (50 ug).

Figure 13: Mutant p53 elutes from a Sp1 DNA affinity column at 0.4M KCl as determined by the p53 antibody 421 induce supershift on an SCS oligo. *Nuclear extracts from MDA MB 468 cells were fractionated on a sephacryl S-300 column to eliminate nucleases. The fractions were combined, concentrated and 1500 ug of protein was passed through a Sp1 DNA affinity column. The column was washed 5 times with buffer Z containing 0.1 M KCl and the bound factors were eluted with increasing concentrations of KCl (0.2M to1.0M). Each of the 1 ml fractions collected for the washes and elutions were concentrated to 0.1 ml. 3 ul of each fraction was used in the EMSA assay. The DNA binding reaction reactions were carried out at room temperature for 20 minutes in a 30 ul reaction volume in a buffer containing 0.1 pmoles of oligonucleotide, 3 ul of the indicated fractions, 20 mM Hepes(pH 7.8), 100 mM KCl, 1mM EDTA, 1mM DTT, 1 ug of sonicated salmon sperm DNA and 10% glycerol in the presence of a p53 antibodies (421) where indicated. 15 ul of each reaction mixture was resolved by electrophoresis on a 4% acrylamide gel at 200V at 4°C. INP indicates input (0.13%). FT is the flow through (0.13%) and W5 corresponds to wash number five (3 ul). The arrow indicates the p53 Ab 421 induced supershift and the "u" is a band corresponding to a unidentified binding factor.*

Figure 14:

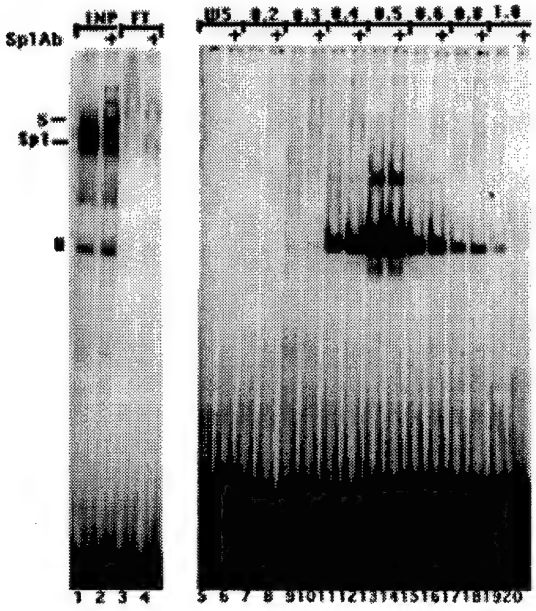


Figure 14: Sp1 elutes from a SCS DNA affinity column in fractions 0.3M and 0.4M as determined by gel mobility assay on an oligonucleotide containing Sp1 binding sites. *Nuclear extracts from MDA MB 468 cells were fractionated on a sephacryl S-300 column to eliminate nucleases. The fractions were combined, concentrated and 1500 ug of protein was passed through a SCS DNA affinity column. The column was washed 5 times with buffer Z containing 0.1 M KCl and the bound factors were eluted with increasing concentrations of KCl (0.2-1.0M). Each of the 1 ml fractions collected from the washes and elutions was concentrated to 0.1 ml. 3 ul of each fraction was used in the EMSA assay. The DNA binding reaction reactions were carried out at room temperature for 20 minutes in a 30 ul reaction volume in a buffer containing 0.1 pmoles of oligonucleotide, 3 ul of the indicated fractions, 20 mM Hepes(pH 7.8), 100 mM KCl, 1mM EDTA, 1mM DTT, 1 ug of sonicated salmon sperm DNA and 10% glycerol in the presence of a Sp1 antibody where indicated. 15 ul of each reaction mixture was resolved by electrophoresis on a 4% acrylamide gel at 200V at 4°C. INP indicates input*

(0.13%). FT is the flow through (0.13%) and W5 corresponds to wash number five (3 ul). The "s" indicates the supershift cause by the sp1 antibody and the "u" is a band corresponding to an unidentified binding factor. Lanes 1-4 are from the same gel as 5-20 but from a different time exposure.

Figure 15:

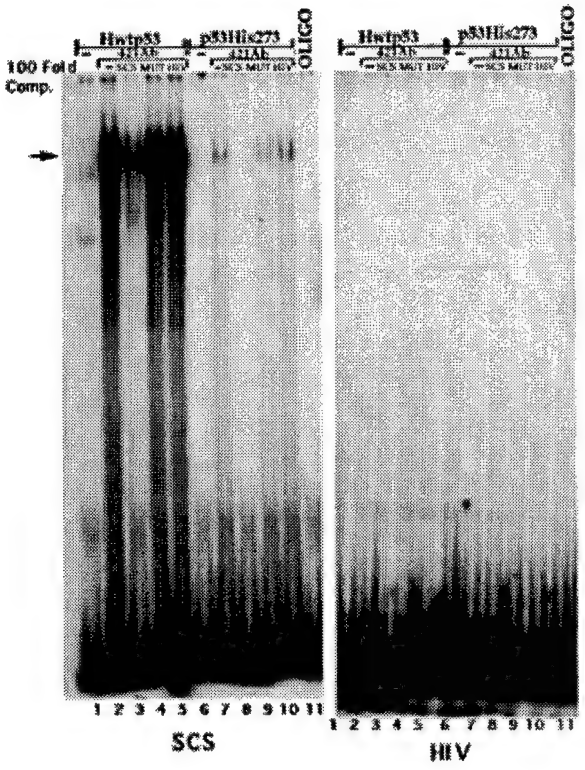


Figure 15: Purified wild type and mutant p53 His 273 bind the SCS oligonucletide but neither purified protein binds the HIV oligonucletotide. *The DNA binding reaction reactions were carried out at room temperature for 20 minutes in 30 ul reaction volume in a buffer containing 0.1 pmoles of oligo, either 23 ng of purified wt p53 or 99 ng of purified mutant, 20 mM Hepes (pH 7.8), 100 mM KCl, 1 mM EDTA, 1 mM DTT, 1 ug of sonicated salmon sperm DNA and 10% glycerol in the presence of a p53 antibody (421) where indicated. 15ul of each reaction mixtures was resolved by electrophoresis on a 4% acrylamide gel at 200V at 4°C. Where indicated, 100 fold competition was carried out with "cold" oligonucleotides. SCS is the p53 super consensus sequence, "mut" is a mutant site that does not bind p53 and HIV is an oligonucleotide containing the Sp1 binding site in the HIV-LTR.*

Task 6 Studies on the affect of p53 on the DNA replication of the double minute chromosomes in the breast cancer cell line MDA-MB 361.

We grew and stocked this cell line however we never initiated the tasks outlined below.

a. Examine the level of MDM-2 gene amplification level in MDA-MB 361 cell lines that express ts-p53val135, both at the permissive and restrictive temperatures.

b. Make and analyze MDA-MB 361 cell fusions with normal breast cells.

c. Carry out in vivo footprinting on synchronous populations of the above cell types.

Conclusions:

The p53 tumor suppressor protein participates in both a G1/S and a mitotic G2/M cell cycle checkpoint as well as being able to induce apoptosis under certain conditions. p53 is activated by DNA damage induced by various chemotherapeutic drugs, including Camptothecin and Bleomycin. p53 functions as a DNA damage checkpoint protein and regulates the cell cycle. The p53 protein may in turn be regulated by the cell cycle under certain conditions. We have determined that 6 hour treatment of the myeloid leukemia cell line (ML-1) with the topoisomerase I targeted chemotherapeutic agent Camptothecin gives a peak increase in the level of p53 coordinate with an overall increase in the p53 specific DNA binding activity. Activation of the endogenous genes Gadd45 and p21/Wafl are also detectable at this time point. Interestingly the DNA damaging Bleomycin analogue, Zeocin, is unable induce Gadd45 expression. Camptothecin rapidly induces apoptosis of ML-1 cells while Zeocin evokes a cell cycle growth arrest. In addition we have observed that Camptothecin treatment of the TR9-7 cell line containing tetracycline-regulated p53 demonstrates Gadd45 expression only in the presence of Camptothecin induced DNA damage while p21/Wafl expression can be activated by p53 in both the presence and absence of DNA damage. While the tetracycline-regulated p53 is able to mediate differential expression of p53 inducible genes the overall p53 level and DNA binding activity remain unchanged in either the presence or absence of DNA damage. Additionally under these conditions we have seen that the Gadd45 p53 DNA binding site is constitutively DNAse I hypersensitive, demonstrating that chromatin remodeling is not controlling this differential expression. Using an antibody specific to p53 phosphorylated at the CK II site we have demonstrated that Camptothecin activates phosphorylation of p53 at serine-389 while Zeocin does not. We then looked to see if phosphorylation of p53 at serine-389 was cell cycle regulated.

It has been suggested that only during S phase can Camptothecin induce the DNA double strand breaks which elevate p53 levels , while Bleomycin type drugs are able to directly damage the DNA and thus induce double strand breaks during any cell cycle stage[4]. Using centrifugal elutriation we have separated exponentially growing ML-1 cells into specific cell cycle populations and have treated these discrete fractions with Camptothecin for 6 hours. Treatment of the cell cycle fractions with Camptothecin demonstrated a clear increase of p53 in all cell cycle fractions along with a concomitant induction of a p53 species phosphorylated at serine-389. The Camptothecin treated cell cycle fractions showed approximately equal sub-G1 DNA content and the DNA binding activity in the cell cycle fractions (observed by EMSA of a p53 super consensus binding site) corresponded to the level of p53 in each fraction. Interestingly the p53 binding activity for a Gadd45 DNA oligonucleotide was enriched in the Camptothecin treated G2/M cell cycle fraction. However the DNA binding activity and level of p53 do not appear to correlate with the percent of apoptotic cells and p53 dependent apoptosis is not cell cycle regulated. We are currently investigating if p53 mediated G1/S and G2/M growth arrest are controlled by differential regulation of p53 during the cell cycle.

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p53 binds to a constitutively nucleosome free region of the mdm2 gene

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The mdm2 oncogene is a p53 responsive gene which contains both a p53 independent and a p53 dependent promoter (P1 and P2 respectively). We have utilized ligation mediated PCR genomic footprinting in order to investigate the intra-nuclear binding of p53 to the mdm2 P2 promoter. The DNase I protection pattern in nuclei from murine cells lacking p53 has been compared to the protection pattern in cells containing a temperature sensitive p53-Val135. At 32°C p53-Val135 assumes a wild-type conformation while at 37°C this p53 is conformationally mutant. We observed clear wild-type p53 dependent protection of the putative p53 response elements (REs) as well as protection of the adjacent TATA box. Interestingly the protection pattern observed with purified wild-type p53 on naked DNA showed less nucleotide sequence protection than the protection observed to be p53 dependent in nuclei. Constitutive DNase I hypersensitivity at both the mdm2 P1 and P2 promoters was detected by indirect Southern blot analysis. DNase I hypersensitivity reflects altered chromatin conformations resulting, most likely, from the absence of nucleosomes. Taken together our findings suggest that the mdm2 P2 promoter is maintained in a nucleosome free state which is pre-primed for transcriptional activation by p53.

Keywords: p53; mdm2; nucleosome; chromatin; transcription

Introduction

p53 is one of the most frequently mutated genes found in human tumors, demonstrating the importance of p53 for the regulation of normal cell growth (Hollstein *et al.*, 1991; Levine *et al.*, 1991). Wild-type p53 is able to activate transcription from specific DNA sequence regions both *in vitro* and *in vivo* (Farmer *et al.*, 1992; Funk *et al.*, 1992). When the site-specific DNA binding ability of p53 is abolished the protein is no longer able to direct the activation of specific growth controlling genes (Vogelstein and Kinzler, 1992; Zambetti and Levine, 1993). It is not surprising therefore that most oncogenic p53 mutations reside in the DNA binding domain of the protein (Bargonetti *et al.*, 1993; Pavletich *et al.*, 1993; Wang *et al.*, 1993) and that these mutations result in reduced p53 sequence specific DNA binding activity (Bargonetti *et al.*, 1991; Chen *et al.*, 1993a; Kern *et al.*, 1991a,b). Wild-type p53 binds to diverse DNA sequences containing two adjacent copies of the consensus 5′-Pu Pu Pu C (A/T) (T/A) G Py Py Py-3′ (el-Deiry *et al.*, 1992; Funk *et al.*, 1992). The co-crystal structure of p53 with DNA has shown that some oncogenic mutants affect the conformation of the protein while others result in amino acid substitutions at residues which are necessary for direct DNA contacts (Cho *et al.*, 1994). All p53 target genes contain versions of the consensus sequence shown above however the location of the consensus relative to the initiation start site differs for each target gene. While many of the p53 induced gene products have been identified to inhibit cell growth by signaling for either growth arrest or apoptosis (including p21/Waf1, Bax and Gadd45), in contrast a growth activating and transforming function has been associated with the product of the mouse double minute 2 (mdm2) gene (reviewed by Ko and Prives, 1996).

The MDM2 protein is able to form a protein–protein interaction with p53 which inactivates the transactivation function of p53 (Brown *et al.*, 1993; Haines *et al.*, 1994; Momand *et al.*, 1992; Oliner *et al.*, 1993; Wu *et al.*, 1993) and also targets p53 for degradation (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997). Overexpression of MDM2 can inhibit the ability of p53 to suppress transformation (Finlay, 1993) as well as inhibit the ability of p53 to evoke cell cycle arrest or induce apoptosis (Chen *et al.*, 1996). Such abrogation of p53 function by MDM2 is one of the ways MDM2 exerts its oncogenic effect (Oliner *et al.*, 1992; Chen *et al.*, 1996). The mdm2 gene contains a p53 dependent promoter (P2) in addition to a p53 independent promoter (P1) (Juven *et al.*, 1993; Perry *et al.*, 1993; Wu *et al.*, 1993). The mdm2 P2 promoter is localized in the first intron of the gene and contains two p53 REs (Juven *et al.*, 1993; Wu *et al.*, 1993; Zauberman *et al.*, 1995). Mdm2 deficiency results in embryonic lethality which can be rescued by deletion of the p53 gene (Montes de Oca Luna *et al.*, 1995; Jones *et al.*, 1995). This demonstrates that the feedback loop between p53 and MDM2 during normal development is critical. Overexpression of MDM2 in tumors is achieved by different mechanisms which include increased transcription (Sheikh *et al.*, 1993), enhanced translation (Landers *et al.*, 1994; He *et al.*, 1994), gene amplification (Oliner *et al.*, 1992; Refenberger *et al.*, 1993) and gene rearrangements (Leach *et al.*, 1993). The fact that wild-type p53 is involved in the control of mdm2 transcription suggests that transcriptional activation from the p53 REs in the mdm2 gene may be differentially regulated from those target genes whose products signal for growth inhibition.

The regulation of p53 binding to different target genes and thus different p53 response elements (p53 REs) has been studied predominantly using naked DNA (el-Deiry *et al.*, 1993; Funk *et al.*, 1992; Juven *et al.*, 1993; Kastan *et al.*, 1992; Ko and Prives, 1996;

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Wang *et al*., 1995). However the cellular transcription machinery is designed to function with chromatin not naked DNA (reviewed in Felsenfeld, 1996; Kingston *et al*., 1996). Recently histone acetylases and histone deacetylases have been shown to regulate transcription by reconfiguring chromatin (Wolffe, 1996; Taunton *et al*., 1996; Brownell *et al*., 1996; Wolffe and Pruss, 1996). The diversity that exists between p53 target genes suggests that the locations of the p53 REs within chromatin may play a role in the differential activation of the target genes. We have investigated the interaction of p53 with the mdm2 P2 promoter in chromatin. Basal transcription of the mdm2 gene occurs from the P1 promoter and not the P2 promoter, and p53 is the only transcription factor which has been identified to transactivate mdm2 (Juven *et al*., 1993; Wu *et al*., 1993; Zauberman *et al*., 1995).

The specific nucleotides which are protected by p53 in either the naked or chromatin localized mdm2 gene have not been determined. Recently one study examining the *in vivo* binding ability of p53 has been reported (Chin *et al*., 1997). However in this study the authors were unable to detect any p53 dependent protection changes at the mdm2 gene. We have examined the *in vitro* interaction of purified p53 with naked mdm2 DNA sequence as well as the intra-nuclear interaction of p53 with the p53 dependent promoter region of the mdm2 gene. We have observed clear intra-nuclear protection by comparing the genomic DNase I protection patterns of the P2 promoter in two different cell lines, a p53-null cell line (10-1) and a temperature-sensitive p53-Val135 overexpressing line (3-4). Nucleosomes are known to repress gene activation by blocking critical DNA sequence elements. Genes localized in repressed chromatin regions can be activated by disruption of nucleosomes positioned over important regulatory regions. In contrast, some genes have regulatory regions which are maintained in a nucleosome-free state allowing the genes to be primed for transcriptional activation (Felsenfeld, 1992). We have shown (using indirect Southern blot analysis in conjunction with ligation mediated PCR footprinting) that the mdm2 promoters are nucleosome free and that chromatin reorganization is not required for the intra-nuclear p53 dependent protection of the mdm2 P2 promoter. This suggests that a control mechanism exists to maintain the mdm2 gene in a configuration which is readily accessible for transcriptional activation.

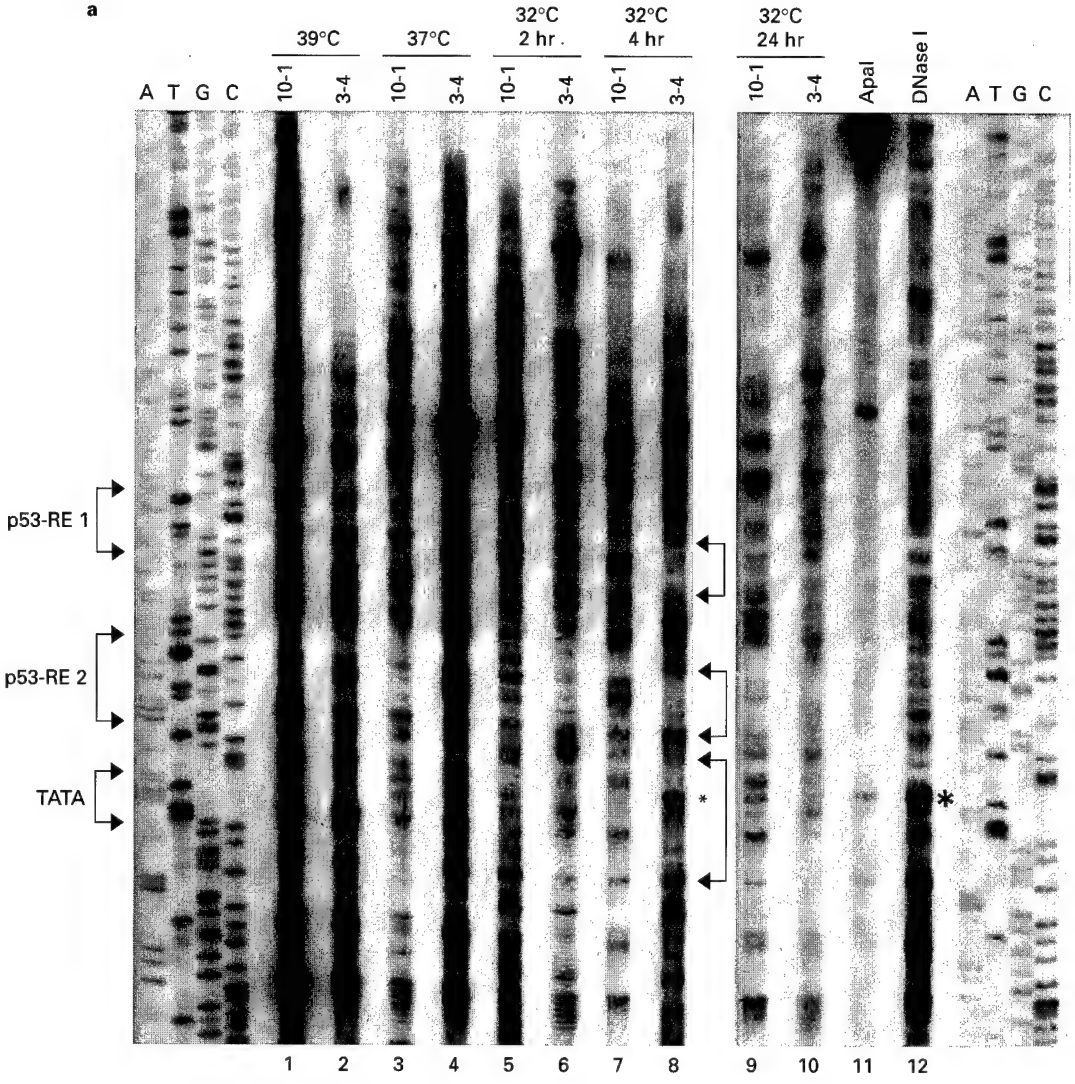
Results

Simultaneous intra-nuclear protection of the p53-REs and the TATA box

Ligation mediated PCR genomic footprinting was used to define the nucleotide sequences in chromatin protected in a p53 dependent manner. We compared the protection pattern at the mdm2 P2 promoter in the p53-null cell line (10-1) to that in the ts p53-Val135 containing cell line (3-4). Striking protection of the p53-REs was observed in nuclei of 3-4 cells maintained at 32°C for 4 h (Figure 1a, lane 8; the protected regions are shown by the brackets on the right while the putative p53-REs are shown on the left). While no changes were observed between the protection patterns at p53 RE-1 or p53 RE-2 in 3-4 cells maintained at 39°C or 37°C, after incubation at 32°C for 4 h the 3-4 cells showed protection of the downstream 3′ halves of both putative p53 binding sites (Figure 1a, compare lanes 2, 4 and 8). After 2 h of incubation at 32°C slight differences in protection of the P2 promoter in the 3-4 cells emerged, but these changes were not as dramatic as those which occurred after 4 h at 32°C (Figure 1a, compare lanes 6 and 8). Additionally, a change in protection at the TATA box region was clear at the 4 h incubation time point (Figure 1a, lane 8 indicated by bracket with *). The DNase I hypersensitive site which emerged (marked *) was also visible in the naked DNA sample (Figure 1a, lane 12) indicating that at the other time points this region may be protected by a factor. It has been shown that p53 and TFIID are able to bind cooperatively to DNA (Chen *et al*., 1993b). The simultaneous change in protection at the p53-REs and the TATA box suggests that binding of p53 facilitates recruitment of TFIID to the promoter region. DNA affinity chromatography using the mdm2 p53 binding site region without the TATA box has demonstrated p53 dependent recruitment of TBP when comparing 10-1 and 3-4 nuclear extracts (Molina and Bargonetti, unpublished) which further supports the idea of p53 mediated TFIID recruitment. The 3-4 cells shifted to 32°C for 24 h showed reduced protection of the two p53-REs with concomitant extended protection over the 3′ adjacent TATA box region (Figure 1a, lane 10). The pattern of genomic protection in 10-1 and 3-4 cells was similar (although not identical) at 39°C and 37°C (Figure 1a, lanes 1–4). Changes in the 10-1 protection pattern were observed at 32°C but these changes did not correlate with the putative p53-REs. The areas of genomic protection evident in the 3-4 cells after 4 h at 32°C overlapped with (but did not completely contain) the predicted p53 sequence recognition elements. Figure 1b shows the oligonucleotides used for LM-PCR footprinting and the mdm2 P2 promoter sequence. The areas of predicted protection are contrasted with the regions which showed p53 dependent changes in the chromatin protection pattern. PhosphorImager analysis of a Northern blot of the mdm2 mRNA produced after 4 h of incubation at 32°C indicated that the protection at the p53-REs and the TATA box correlated with a 16-fold increase in transcription (Figure 2).

p53 mediated DNase I protection of the mdm2 p53-REs is different on naked DNA

We examined the *in vitro* DNase I footprinting ability of purified p53 on the mdm2 P2 promoter region of naked DNA in order to determine if the pattern of protection at the p53-REs was the same *in vitro* and *in vivo* (Figure 3). Surprisingly, the *in vitro* protection pattern demonstrated with purified p53 on naked DNA differed considerably from the p53 dependent genomic protection pattern. Immunopurified p53 on naked DNA protected the 5′ half of p53-RE1 and showed no clear protection of p53-RE2, although an adjacent area of protection was identified (Figure 3, lanes 1 and 2). The footprinting pattern with purified p53 demonstrated a pattern of protection in which



b

ApaI

(1bp)gggcclcgctccgggtcgcgctggctcgttgctgggtccaggag
gtgacaggtgcctggtcccggactcgccgggatgcggcttccgggacgg
gtgggactggtctgggccgagttgactcagctcttcctgtggggct**ggt**
caagttgggacacgtccggcgtcggctgtcggaggagctaagt
p53 RE-1
cctgacatgtctccagctggggttat*ttaaacgctgccccg
p53 RE-2 **TATA box**
tttccgcagccgtctgctgggcgagcgggagaccgaccggacacccctg
ggggaccctctcggatcaccgcgcttctcctgcgggcctccaggtaagg
agcagctcgccgacgtcgttttgcatttgagagctattgccgaaagacgtt
NsiI
ttctgctccttcgtaaatlgcatgtatctatttgtccctttcgtagatgtttt
ataatttcaagtttttatcgtgtgtgttgttgttttttttctacttgtaggcca
atgtgcaataccaacatgtctgtgtctaccgagggg*tgctgcaagcacctca*
oligo#3 ====

*cagattccagcttcg*gaacaagagactct*ggttggtatttctacctcga*
======= oligo #2 oligo #1

-(585bp)

Figure 1 Ligation-mediated PCR *in vivo* footprinting demonstrates p53 mediated protection of the p53-REs. (**a**) The published p53-REs and adjacent TATA box were identified by sequencing the subcloned genomic mdm2 (a generous gift from Donna George) (lanes are indicated as ATGC). The DNase I treated samples were prepared from nuclei of either 10-1 or 3-4 cells maintained at 39°C (lanes 1 and 2), 37°C (lanes 3 and 4), 32°C for 2 h (lanes 5 and 6), 32°C for 4 h (lanes 7 and 8) and 32°C for 24 h (lanes 9 and 10). Purified DNA from undigested nuclei is shown in lane 11 and naked mouse genomic DNA digested with DNase I is shown in lane 12. Ligation mediated PCR was carried out followed by primer extension with ^{32}P-labeled oligonucleotide #3 which hybridized approximately 350 bp downstream from the P2 promoter. Samples were electrophoresed on a 6% urea sequencing gel. (**b**) Sequence representation of the mdm2 P2 promoter region showing areas of predicted protection overlapping with areas protected during footprinting. The large bold letters indicate protection as seen during genomic footprinting. The double underlined regions depict areas that were the predicted binding elements. The asterisk represents the location of hypercutting found in both the 3-4 32°C 4 h sample (lane 8) and in the DNase I digested naked genomic DNA sample (lane 12). Oligonucleotides used for ligation mediated PCR are numbered and shown in underlined italics. The overlap between oligonucleotides #2 and #3 is denoted by the dashed underline

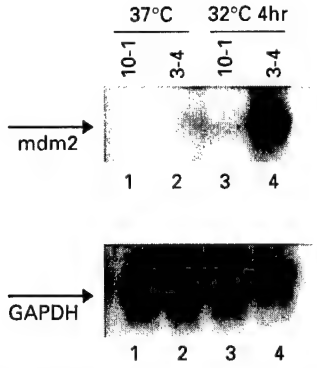


Figure 2 Endogenous levels of mdm2 RNA are enhanced by the presence of wild-type p53 activity. Northern blot analysis of mdm2 poly(A) RNA in 10-1 cells (lanes 1 and 3) and in 3-4 cells with the temperature sensitive p53 Val135 (lanes 2 and 4). Cells were grown at 37°C and then shifted to 32°C for 4 h. The blot was hybridized first with *NsiI–ApaI* mdm2 probe then stripped and reprobed with a GAPDH fragment. Data were also quantitated from multiple exposures on a PhosphorImager

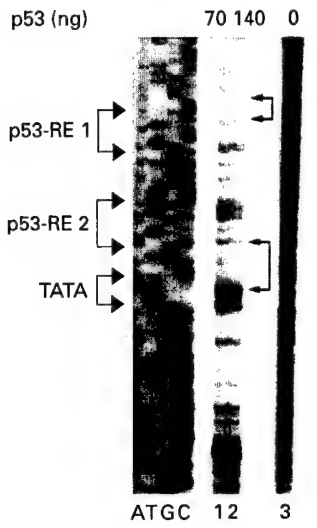


Figure 3 DNase I protection of the p53-REs of mdm2 *in vitro* differs from that observed *in vivo*. *In vitro* footprinting of a naked mdm2 DNA fragment with purified p53. The published p53-REs and adjacent TATA box were identified by sequencing genomic mdm-2 in a plasmid clone (lanes indicated as ATGC) and are indicated on the left. Increasing amounts of purified p53 were added to a complete *ApaI–XhoI* digest of the naked mdm2 genomic plasmid clone (lanes 1 and 2, as indicated). Footprints were visualized by primer extension using ^{32}P-labeled oligonucleotide #3 which hybridized 350 bp downstream from the TATA box. Lane 3 contains no p53 protein

sequences extending from the specific site were alternately protected and hypersensitive to DNase I cutting; this is consistent with our previous observations with purified p53 at other p53 binding sites (Bargonetti *et al.*, 1992). The auxilary protection may be a result of p53 protein multimers wrapping around the DNA (Stenger *et al.*, 1994). The difference between the genomic footprinting pattern and the *in vitro* protection pattern suggests that the chromatin structure of the mdm2 gene organizes the P2 promoter region of DNA into a sequence which is an optimal p53 DNA binding site. Another possibility for the increased binding of p53 to chromatin is that post-translational modifications of p53 in the nucleus facilitated p53 binding to the genomic mdm2 chromatin.

The mdm2 P1 and P2 promoters are constitutively nucleosome free

When genes controlled by specific promoters are transcriptionally active, or are primed to be activated, their promoter regions in chromatin are marked by increasing sensitivity to DNase I which results from disruption of nucleosomes (Gross and Garrard, 1988; Weintraub and Groudine, 1976). We took advantage of this fact and screened for the appearance of p53 dependent DNase I hypersensitive sites at the mdm2 P2 promoter. We examined by indirect end-labeling analysis the genome from isolated DNase I treated nuclei of 10-1 or 3-4 cells maintained at 37°C, 32°C for 4 h or 32°C for 24 h (Figure 4a–c). Interestingly the same two DNase I hypersensitive sites were evident in both the 10-1 and 3-4 cell lines at the different temperature shift points indicating that p53 did not induce nucleosome positioning changes. One hypersensitive site was observed 2.2 kb from the *Eco*RI cutting site which corresponded to the mdm2 P1 promoter region (Figure 4a, indicated by the P1 arrow) while the other was observed 1.5 kb from the *Eco*RI cutting site which corresponded to the location of the P2 promoter region (Figure 4a, indicated by the P2 arrow). The overall DNase I sensitivity of these two sites did not change in the presence of wild-type p53 over the course of a 24 h temperature shift to 32°C (Figures 4b and c). The constitutive nature of the hypersensitive site at the mdm2 P2 promoter indicated that this region was maintained as an altered chromatin structure in the absence of p53 (Figure 4a). To further analyse the nucleosome arrangement we assayed the micrococcal nuclease sensitivity of the p53 dependent mdm2 P2 promoter. Although ethidium bromide staining of the agarose gel showed the expected nucleosome fragmentation ladder Southern blotting of both the 10-1 and 3-4 genomes with a P2 promoter specific probe gave a barely detectable smearing signal without a nucleosome ladder (data not shown). Taken together these results further support that the P2 promoter region was constitutively nucleosome-free.

Increased p53 protein levels correlate with the genomic footprint

Previous reports on the properties of ts p53-Val135 have shown increased levels of nuclear p53 protein when the cells are shifted to 32°C (Martinez *et al.*, 1991). In order to determine if the genomic protection of the mdm2 p53-REs corresponded to an increase in nuclear p53 we analysed the nuclear level of p53 at the different temperature shift time points (Figure 5). A twofold increase in the nuclear p53 level was observed when the cells had been maintained at 32°C for 4 h (Figure 5, lane 8) and a decrease in the level of p53 was observed after 24 h at 32°C (Figure 5, lane 10). This decrease in p53 from the 4 h time point to the 24 h time point was undoubtably due to the documented short half-life of the protein (Ginsberg *et al.*, 1991) and

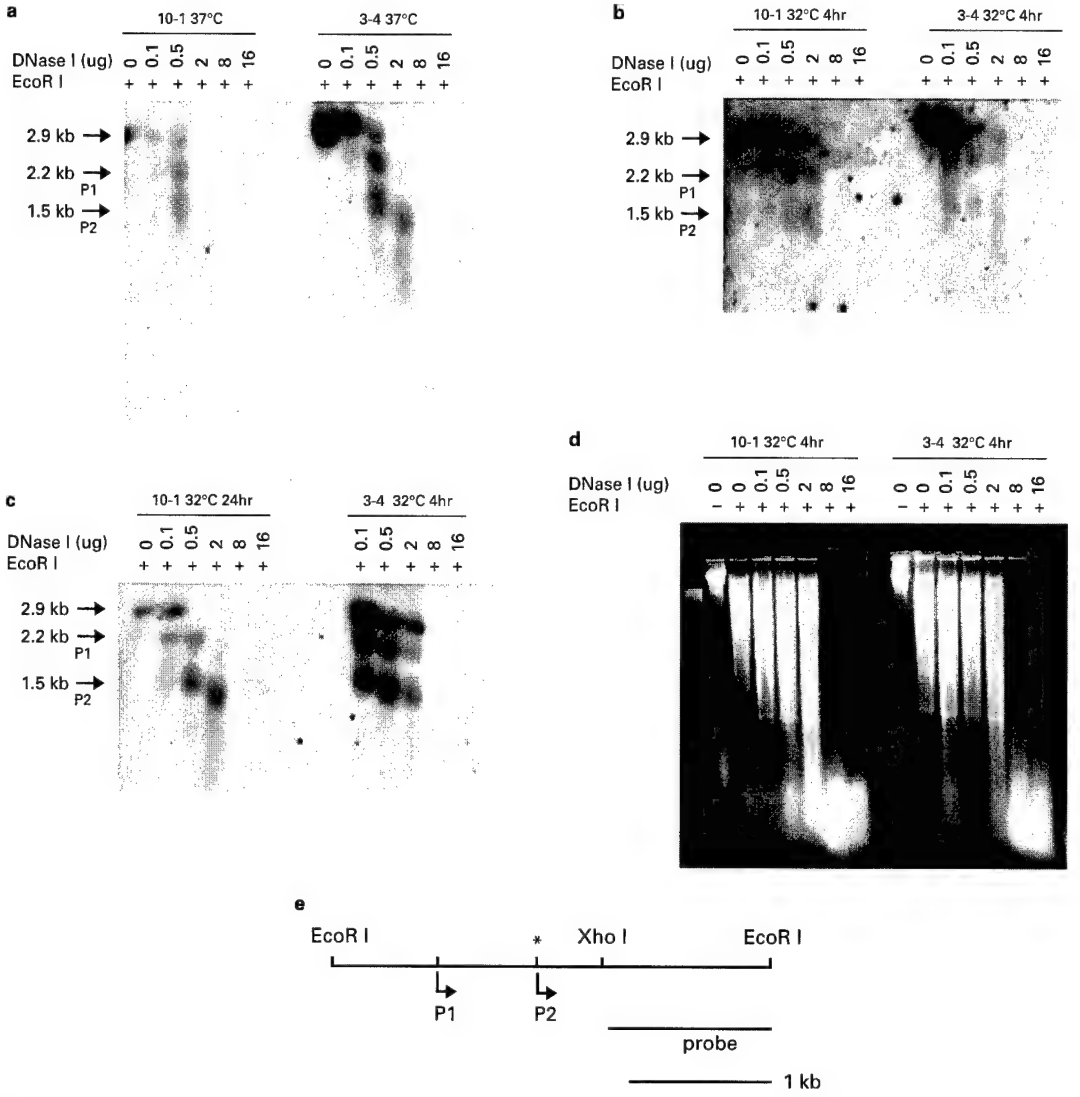


Figure 4 The mdm2 P1 and P2 promoters are constitutively nucleosome free. DNA in 2×10^6 isolated nuclei was digested with increasing amounts of DNase I (0 μg, 0.1 μg, 0.5 μg, 2 μg, 8 μg or 16 μg as indicated above each lane). Nuclei were isolated from 10-1 and 3-4 cells maintained at 37°C (**a**), 32°C for 4 h (**b**) or 32°C for 24 h (**c**). The ethidium stained agarose gel of **b** is also shown (**d**). Purified DNA was restricted with *Eco*RI and electrophoresed on a 0.8% agarose gel, probed with a ^{32}P-labeled *Eco*RI–*Xho*I genomic mdm2 probe fragment (shown in **e**). The arrows indicate the hypersensitive sites in the mdm2 gene. (**e**) Physical map of the upstream region of the murine mdm2 gene (Barak *et al*., 1994). The * delineates the location of the p53 REs

at least partially explains the dynamic intranuclear footprint that was observed. All lanes on the gel were normalized for the same amount of total protein. We subsequently demonstrated equivalent levels of protein in all samples by stripping the gel and Western blotting with an antibody to the ubiquitous transcription factor Sp1 (data not shown). Interestingly, this increased level of nuclear localized wild-type p53, in addition to the concomitant nuclear chromatin binding did not initiate additional DNase I hypersensitive sites (Figure 4b).

EMSA with nuclear extract containing p53-Val135 shows possible p53 mediated recruitment of other factors to the mdm2 DNA site

It has been demonstrated that p53 can bind differentially to p53-REs that contain variations of the p53 consensus binding sequence. Phosphorylation of the C-terminal end of p53 as well as addition of the p53 specific antibody PAb421 are able to activate p53 DNA binding activity (Hupp *et al*., 1992; Funk *et al*., 1992; Halazonetis *et al*., 1993; Hecker *et al*., 1996; Takenaka *et al*., 1995; Wang and Prives, 1995). We examined the p53 dependent gel shift of an oligonucleotide containing the mdm2 P2 p53-REs using a standard electrophoretic mobility shift assay (EMSA). No p53 dependent gel shift species were observed in either the absence or presence of the p53 specific antibody PAb421 when examining nuclear extract from 10-1 or 3-4 cells maintained at 37°C (Figure 6a, compare lanes 1 and 2 to lanes 7 and 8). A striking p53 dependent gel shift of the mdm2 oligonucleotide was observed in the presence of PAb421 with nuclear extract derived from 3-4 cells maintained at 32°C for 4 h, while the corresponding 10-1 nuclear extract showed no p53 dependent gel shift species (Figure 6a,

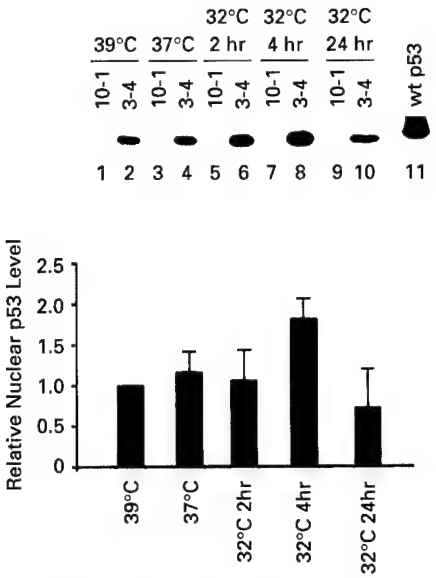


Figure 5 Nuclear p53 protein levels in 10-1 and 3-4 cells. Nuclear extracts were prepared as described in the Materials and methods. 100 μg of total protein was resolved by electrophoresis on a 10% SDS–PAGE. The p53 in samples was visualized by Western blotting with a mixture of p53 specific monoclonal antibodies PAb 421, PAb 240 and PAb 1801 and detection with ECL reagent (Amersham). The relative levels of p53 are represented as a histogram of normalized pixel values determined by laser densitometer analysis and quantitated by Image QuanNT software (version 4.1) setting the level detected at 39°C as 1. The standard deviation for all other samples was calculated from three experiments

compare lanes 3 and 4 to lanes 9 and 10; the shift is indicated by the arrow on the left). Nuclear extract derived from cells maintained at 32°C for 24 h gave a reduction in the p53 dependent gel shift species and this also corresponded to the decreased level of p53 protein shown by Western blot analysis (Figure 6a, compare lanes 10 and 12). The gel shift complex observed in the presence of immunopurified p53 and PAb421 migrated faster than the species from crude nuclear extracts (Figure 6b). It is possible that the different source of p53 influenced the gel migration. However a comigrating PAb421 induced supershift species was evident from reactions containing 3-4 nuclear extract and purified p53 when a super consensus site sequence (SCS) oligonucleotide was used for the EMSA (data not shown). Together these data suggest that either additional proteins from the nuclear extract sample are complexed with the mdm2 oligonucleotide or that different post-translationally modified forms of p53 bind to the SCS and mdm2 oligonucleotides.

A competition gel shift assay was carried out to further support the specificity of the p53 binding to the mdm2 oligonucleotide. The PAb421 induced supershift from the 32°C 4 h nuclear extract sample was competed away with both non-radioactive mdm2 oligonucleotide as well as with an oligonucleotide containing the p53 super consensus site sequence (SCS) (Figure 6b, lanes 1–5) while a mutant p53 binding site oligonucleotide was unable to compete away this p53 dependent gel shift species (Figure 6b, lanes 6 and 7). This result was analogous to that

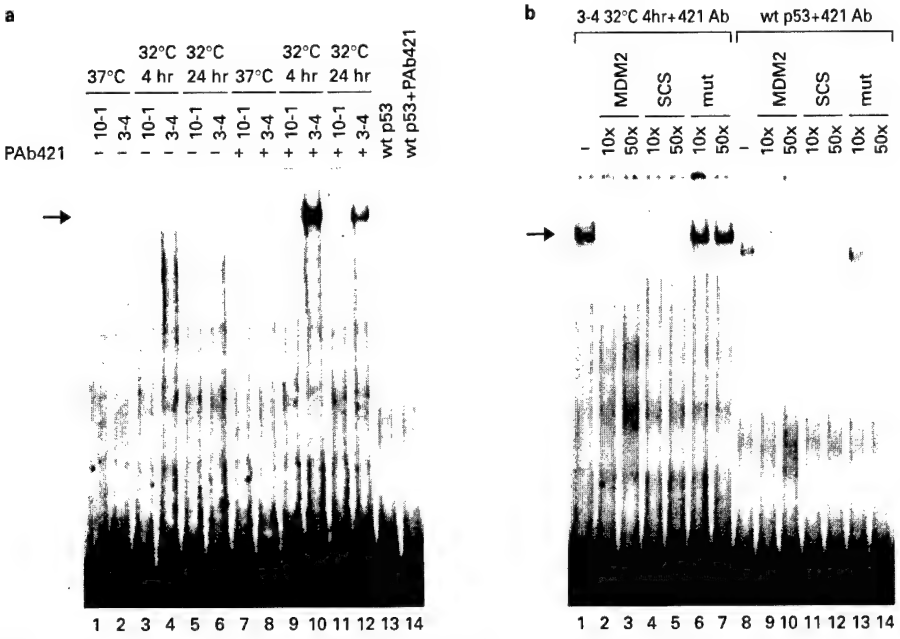


Figure 6 Protection of the mdm2 P2 promoter *in vivo* corresponds to the enhanced binding of nuclear p53 to an mdm2 P2 oligonucleotide. **(a)** EMSA of a mdm2 P2 containing oligonucleotide with 2 μg of 10-1 (lanes 1, 3, 4, 5, 7, 9 and 11) or 3-4 nuclear extracts (lanes 2, 4, 6, 8, 10 and 12) in the absence (lanes 1–6) or presence of PAb 421 (lanes 7–12). Lanes 13 and 14 contain purified wild-type p53. **(b)** EMSA of mdm2 P2 containing oligonucleotide with 2 μg of 3-4 nuclear extracts (lanes 1–7) and 150 ng of purified wild-type p53 (lanes 8–14) in the presence of PAb 421. Competition was carried out with 10 and 50 fold excess of either mdm2 oligonucleotide (lanes 2, 3, 9 and 10), p53 super consensus sequence (lanes 4, 5, 11 and 12) or mutant RGC oligonucleotide (lanes 6, 7, 13 and 14). The arrows indicate the nuclear extract derived p53 dependent gel shift species

obtained using immunopurified wild-type p53 (Figure 6b, lanes 8–14); except for the fact that the samples containing immunopurified p53 produced a markedly faster migrating gel shift species in EMSA than the nuclear extract.

Discussion

Studies on the ability of p53 to bind to DNA have been carried out predominantly *in vitro*, but little is known about the ability of the protein to bind to specific target sites *in vivo* (Chin *et al.*, 1997). The region of the mdm2 gene with which p53 interacts was originally identified by co-immunoprecipitation of a specific DNA element as well as by demonstrating a minimal sequence able to confer p53 specific transactivation of a reporter construct (Juven *et al.*, 1993; Wu *et al.*, 1993). The nucleotides within this region that are specifically bound by p53 and thereby protected from DNase I digestion were not identified. Using ligation mediated PCR intra-nuclear footprinting, we have shown clear protection of sequences within the p53 REs in the mdm2 gene. This protection was dynamic and occurred after a 4 h 32°C temperature shift of the 3-4 cells. A 16-fold activation of mdm2 transcription was also noted at this time point. Therefore, it is not surprising that the footprinting pattern demonstrated protection of the TATA box region as well. It is clear that the pattern of protection at both the p53-REs and the TATA box region showed dramatic differences in the 3-4 cells maintained at 32°C for 4 h when compared to the 3-4 cells maintained at 39°C or 37°C. Interestingly the *in vitro* p53 protection pattern of the naked mdm2 DNA sequence did not correlate well with the putative p53 REs. Immunopurified p53 protected only one of the two p53 REs on naked DNA. p53 dependent intra-nuclear protection, on the other hand, showed a correlation between the protected regions and the two putative p53 REs. There are a number of possible explanations for these results. First the chromatin structure of the mdm2 gene may facilitate the binding of p53. Second, post-translational modifications of p53 in the nucleus may facilitate the binding of p53 to the mdm2 sequence. Finally, protein–protein interactions may be functioning to recruit p53 to the mdm2 chromatin region.

The p53 consensus DNA binding sequence consists of two functional half-sites (el-Deiry *et al.*, 1992; Wang *et al.*, 1995). These half sites can be separated by 0–13 bp but p53 binds separated half sites best when they are centered on the same face of the DNA helix (Wang *et al.*, 1995). The p53-DNA crystal structure indicates that the core domain binds primarily to a single pentamer consensus (ie. Pu Pu Pu C A/T) (Cho *et al.*, 1994). The mdm2 P2 promoter in both humans and mice contains two consensus p53-REs and each has two contiguous half sites (i.e. four pentamers) (Zauberman *et al.*, 1995). Thus the fact that the protection pattern does not completely cover both p53 REs is not surprising. Interestingly, deletion of one of the mdm2 p53-REs virtually abolishes the ability of p53 to transactivate a promoter reporter construct (Zauberman *et al.*, 1995). It has been suggested that the mdm2 P2 promoter region may contain two relatively weak p53 binding sites as a means of preventing premature activation (Zauberman *et al.*, 1995). DNA binding by p53 oligomers as well as p53 mediated DNA looping are linked with transcriptional activation (Stenger *et al.*, 1994). DNA looping at the mdm2 P2 promoter may be facilitated (and necessary) in the presence of the two p53-REs by using a half site from each. The fact that the nuclear extract derived p53-mdm2 oligonucleotide gel shift species migrated more slowly than the purified p53 gel shift species suggested that other nuclear proteins were associated with the p53-DNA complex. The difficulty obtaining a clear *in vitro* footprint of the mdm2 p53 REs may have resulted because a specific chromatin configuration is needed to facilitate p53 binding. Extended areas of protection and hypercutting resulted in the presence of purified p53 which may have been due to the p53 wrapping around the DNA in the absence of other nuclear proteins. It is possible that p53 requires additional nuclear proteins in order to efficiently bind to the mdm2 DNA sequence. TFIID and p53 bind cooperatively to DNA (Chen *et al.*, 1993) and the redox protein Ref-1 has been shown to activate the DNA binding ability of p53 (Jayaraman *et al.*, 1997). The mdm2 P2 promoter may be an example of a promoter where p53 will bind efficiently only in the presence TFIID and other protein factors. We are investigating this possibility.

Although the ligation mediated footprint resulting in the 3-4 cells maintained at 32°C for 24 h did not show protection of the p53 REs, some p53 dependent DNA binding was observed by EMSA with nuclear extract derived from these cells. This suggests that p53 may be tethered to the complex via association with other DNA bound proteins. In a recent study the relative instability of p53-DNA interactions *in vivo* has been noted (Chin *et al.*, 1997). In fact the authors were unable to explain their complete lack of an *in vivo* footprint at the mdm2 gene. The reason why we were able to see a protection pattern and they were not may be explained by the different cell systems used. In the case of the murine cell line used for the experiments presented here the MDM2 levels must be maintained low enough for the cells to undergo a cell cycle arrest (as this is the phenotype for the 3-4 cells at 32°C). Alternatively regulatory mechanisms may exist to maintain p53 activity which functions to arrest cell growth. The human ML-1 cell line used by Chin *et al.*, normally undergoes apoptosis in response to the activation of wild-type p53 by DNA damage (Nelson and Kastan, 1994). The p53 in the 3-4 cell line and the p53 in the ML-1 cell line may contain different post-translational modifications.

Constitutive hypersensitive sites are often present in promoter regions of genes poised for transcriptional activation and these sensitive sites exist independent of gene expression (Gross and Garrard, 1988). Using DNase I hypersensitive site mapping of the mdm2 gene we have identified two DNase I hypersensitive sites which localized at both the P1 and P2 promoters of the mdm2 gene. Chromatin structure has been shown to regulate gene expression. The data presented here show that the mdm2 gene is a member of the class of genes which exist in a preferentially DNase I sensitive state. This suggests that the mdm2 gene is primed for both transcriptional activation and repression (Gross and Garrard, 1988). The two DNase I hypersensitive sites

occurred in both the presence and absence of p53. No changes in mdm2 DNase I hypersensitivity resulted when p53 bound to the P2 promoter. Our results suggest that a mechanism exists in order to maintain the mdm2 gene in a configuration which is readily accessible for the binding of protein factors, one of which is p53.

p53 is a nuclear phosphoprotein and the DNA binding activity of p53 can be activated by phosphorylation of the protein's C-terminal domain (reviewed in Ko and Prives, 1996). Recently it has been shown that the C-terminal domain of p53 can be acetylated by its co-activator p300 and that this posttranslational modification activates the DNA binding activity of p53 (Gu and Roeder, 1997). Interestingly p300/CBP and p53 can synergistically activate transcription of a transiently transfected construct containing the mdm2 P2 promoter (Gu *et al*., 1997). The DNase I sensitivity studies presented here demonstrate that activation of the mdm2 gene does not require chromatin remodeling. Taken together these findings suggest that the acetyltransferase p300 activates p53-mediated mdm2 transcription by direct acetylation of p53 and not via the modification of histones. In light of the fact that p53 and p300 co-localize within the nucleus and co-exist in a stable DNA-binding complex (Lill *et al*., 1997) it is possible that the acetyltransferase p300 functions differently at a wide array of p53 target sites dependent upon the chromatin configuration of each gene.

It has been reported that chain elongation by RNA polymerase II is unimpeded by the presence of one or two histone octamers in its path *in vitro*. When longer arrays of octamers are present, pol II is still able to elongate RNA *in vitro*, but transcription is partially inhibited by pausing along the template. This effect may well be alleviated *in vivo* by histone modification or the presence of additional factors (Felsenfeld, 1992). Since we did not find other DNase I hypersensitive sites downstream of the mdm2 gene TATA box in either the 32°C 4 h or 24 h samples, we would like to suggest that drastic disruption of the nucleosomes does not occur during elongation. Our results suggest that the chromatin structure of the mdm2 gene may be important for regulation of gene expression by p53 and other factors. This would not be surprising in light of the fact that the mdm2 gene is an essential gene for mouse development and thus transcriptional activation and repression of the mdm2 gene must be rapidly coordinated (Montes de Oca Luna *et al*., 1995). It is possible that nucleosome free chromatin structures are not present in all p53 REs. For example the chromatin remodeling histone transacetylase p300/CBP has been shown to bind to and modulate p53 activity at both the p21/WafI and Bax DNA binding elements (Lill *et al*., 1997; Avantaggiati *et al*., 1997) and the p53 response elements of these genes may be localized in repressed chromatin. In p53 regulated genes where the protein product negatively regulates cell cycle progression nucleosome disruption and chromatin remodeling may be required as a part of the transcriptional control mechanism; this would most likely involve the recruitment of a histone transacetylase. Most of the identified genes activated by p53 negatively regulate cell growth while mdm2, in contrast, positively regulates cell cycle progression. We postulate that chromatin structure is one of the mediators which regulates p53 recognition of binding sites during the course of the cell cycle and therefore chromatin structure may act as a modulator of p53 driven gene activation. Further studies are in progress to investigate these possibilities.

Materials and methods

Cells and viruses

The 10-1 cell line is a mouse fibroblast cell line lacking endogenous p53 protein due to a deletion of the p53 gene (Martinez *et al*., 1991). 10-1 cells were co-transfected with the temperature-sensitive mutant p53-Val135 plasmid (pLTRp53cGval135) (Michalovitz *et al*., 1990) and a Neomycin resistant plasmid to create a stable p53 expressing cell line called 3-4 (Chen *et al*., 1995). Spodoptera frugiperda cells (Sf21 cells) and the recombinant baculoviruses expressing wild-type human p53 were as described (O'Reilly and Miller, 1988). Sf21 cells were grown at 27°C in TC-100 medium (GIBCO) containing 10% heat-inactivated fetal bovine serum.

DNase I treatment of nuclei

10-1 and 3-4 cells were grown on 150 mm plates at 37°C in Dulbecco's Modified Eagle Medium (GIBCO) containing 10% heat-inactivated fetal bovine serum (GIBCO) until 80% confluence. Sub-confluent plates were shifted to either 39°C for 18 h, 32°C for 4 h or 32°C for 24 h. The cells were washed twice with 4°C 1 × PBS, scraped off the plate and spun down at 2500 r.p.m. (4°C) for 10 min. The pellet was resuspended in 2 ml of RSB per plate (10 mM Tris-HCl pH 7.4; 10 mM NaCl; 3 mM $MgCl_2$ pH 7.4; 0.5% NP-40) with 1 mM PMSF. The cells were homogenized 30-40 strokes and checked by trypan blue exclusion. Nuclei were isolated by centrifugation at 4000 r.p.m. at 4°C for 10 min and washed once with RSB. 2.0×10^6 nuclei in 250 μl of RSB were treated with increasing amounts of DNase I (Worthington; 2932 units/mg) for 10 min at either 37°C or 32°C. The reactions were stopped by adding 250 μl of DNase I stop buffer (2 M NH_4OA_c; 100 mM EDTA; 0.2% SDS) followed by proteinase K digestion overnight at 37°C at a final concentration of 400 μg/ml. The DNA was extracted once with phenol and three times with chloroform followed by ethanol precipitation.

DNA and RNA blot analysis

20 μg of DNase I treated genomic DNA was digested to completion with *Eco*RI (GIBCO). The digested DNA was electrophoresed on a 0.8% agarose gel (GIBCO) overnight at 23 V and transferred to positively charged nylon membrane (Schlelcher and Schuell) by electro-transfer at 0.1 Amp overnight at 4°C. Cytoplasmic RNA was isolated from 10-1 and 3-4 cells according to Maniatis. Poly(A) $^+$RNA was prepared using mini-oligo dT spin columns (5′ Prime→3′ Prime, Inc.). 1.5 μg of poly(A) $^+$RNA from each sample was electrophoresed on a 1% formaldehyde-agarose gel and transferred onto positively charged membrane as described above. The membranes were baked at 80°C for 2 h and U.V. crosslinked. The murine mdm2 1.1 kb probe for Southern blot was obtained as a *Eco*RI-*Xho*I fragment from a genomic subclone (Juven *et al*., 1993). The murine mdm2 400 bp probe which contain exon 2 was digested with *Nsi*I-*Apa*I for Northern blot. The GAPDH probe was obtained as a 1.25 Kb cDNA *Pst*I fragment (Marty *et al*., 1985). All probes were labeled with ^{32}P-dCTP by

random prime labeling (Boehringer Mannheim). The specific activity for all the probes used was at least 10^8 c.p.m./μg.

Ligation-mediated PCR for in vivo *footprinting*

The ligation-mediated PCR footprinting technique carried out was an adaptation of that first described by Mueller and Wold (1989) and later revised by (McPherson *et al.*, 1993). The first step of the reaction was carried out in a volume of 15 μl containing 1 μg of genomic DNA derived from nuclei treated with 0.1 μg of DNase I, 1 × Sequenase buffer 1 (25 mM Tris-HCl pH 7.5; 80 mM NaCl; 0.5 mM $MgCl_2$) and 1 μl of 0.3 pmol/μl oligo #1 (5′-TCGAGGTAGAAATACCAACC-3′). The mixture was denatured at 95°C for 5 min and annealed at 50°C for 30 min then chilled on ice. 9 μl of Sequenase buffer 2 (40 mM Tris-HCl pH 7.5; 5 mM $MgCl_2$; 20 mM DTT; 0.1 mM dNTPs) and 0.5 μl Sequenase DNA Polymerase, version 2.0 (USB, 13 U/μl) were then added and the mixture was incubated at 37°C for 10 min followed by inactivation of the enzyme at 68°C for 10 min. The ligation was carried out by adding 20 μl of Ligase buffer 1 (80 mM Tris-HCl pH 7.5; 180 μg/ml BSA; 30 mM $MgCl_2$, 20 μl of Ligase buffer 2 (12 mM ATP; 70 mM DTT), 5 μl Linker solution (20 pmol/μl of 5′-GAATTCAGATC-3′ (LMP-T1) and 3′-CTTAAGTCTAGAGGGCCCAGTGGCG-5′ (LMP-B1) in 250 mM Tris-HCl pH 7.5; 5 mM $MgCl_2$) and 3 μl of T4 DNA Ligase (GIBCO, 1 Weiss unit/μl) followed by incubation at 20°C overnight. The DNA was ethanol precipitated and resuspended in 14 μl of dH_2O. Additional components were then added for PCR reactions as follows: 16 μl of 2.5 mM dNTP, 2 μl of 5 pmol/μl oligo #2 (5′-CGAAGCTGGAATCTGTGAGG-3′), 2 μl of 5 pmol/μl LMP-T1, 1 μl of 4 mg/ml BSA, 4 μl of 10 × Taq buffer (650 mM Tris-HCl pH 8.8; 100 mM β-mercaptoethanol; 165 mM $(NH_4)_2SO_4$; 15 mM $MgCl_2$) and 0.5 μl of Taq DNA polymerase (Boehringer Mannheim 5 U/μl). The samples were cycled 25 times by denaturation at 94°C for 1 min, annealing at 65°C for 2 min and extension at 74°C for 3 min. The PCR products were prepared for visualization using polynucleotide kinase (New England Biolabs) [^{32}P] radiolabeled oligo #3 (5′-GGAATCTGTGAGGTGCTTGCAGCA-3′) for primer extension. 1.5 μl of [^{32}P] oligo #3 (1 pmol/μl) was added to 15 μl of PCR reaction product along with 0.5 μl of 10 × Taq buffer, 3 μl of 2.5 mM dNTPs and 0.5 μl Taq DNA polymerase. Seven PCR cycles of denaturation at 94°C for 1 min, annealing at 68°C for 2 min and extension at 74°C for 3 min were carried out and then 30 μl stop mixture (24 μl TE; 1 μl of 5 mg/ml tRNA; 5 μl of 3M N_aOAc) was added, followed by chloroform extraction and ethanol precipitation. The pellet was resuspended in 12 μl of sequencing loading buffer and the sample was electrophoresed on a 6% denaturing polyacrylamide gel. DNA sequencing reactions were carried out using oligo #3 and Sequenase DNA Polymerase, version 2.0 (USB, 13 U/μl).

Western blot analysis Nuclear lysates were prepared from cells maintained as described above. The cells were washed two times with 4°C 1 × PBS. Cytoplasmic lysis buffer was prepared with 8.8 ml of lysis buffer stock (20 mM HEPES pH 7.5, 20% Glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA pH 8.0, 0.1% Triton X-100, 1 mM DTT, 1 mM PMSF, 50 μg/ml aprotinin, 50 μM leupeptin) and 6.2 ml of ddH_2O. Two milliliters of cytoplasmic lysis buffer were added to each washed 150 mm plate. The cells were scraped off the plate and spun at 2300 r.p.m. at 4°C for 7 min. The supernatant was removed and the pellet was resuspended in 1 ml of nuclear extraction buffer (prepared with 8.8 ml of lysis buffer stock, 0.5 M NaCl, and 4.7 ml of ddH_2O). The cells were rocked with the nuclear extraction buffer for 1 h at 4°C and then centrifuged at 14 000 r.p.m. for 5 min to extract the nuclear proteins. The supernatant was removed and the pellet was discarded. Protein concentrations of the extracts were determined via Bradford Microassay (BIORAD). Samples were electrophoresed on a 10% SDS–PAGE and electro-transferred to nitrocellulose. The blot was probed with a mixture of monoclonal antibodies specific to p53 (PAbs 1801, 240 and 421) and after incubation with goat anti-mouse secondary antibody was visualized using ECL solutions (Amersham).

Purification of p53 proteins Sf21 cells (2.5×10^7/150 mm dish) were infected with recombinant baculovirus and harvested 48 h post infection. Extracts of infected cells were prepared and p53 proteins were purified from cell lysates by PAb421 immunoaffinity procedures as described (Bargonetti *et al.*, 1992). Two different preparations of protein were produced one was eluted with ethylene glycol and the other was eluted with p53 peptide (KKGQSTSRHKK-OH) (Bargonetti *et al.*, 1993); both were dialyzed into a buffer containing 10 mM HEPES (pH 7.5), 5 mM NaCl, 0.1 mM EDTA, 1 mM DTT and 50% glycerol. Ethylene glycol eluted protein was used for EMSAs and peptide eluted protein was used for *in vitro* footprinting.

EMSA The MDM2 synthetic oligonucleotide used in this study, obtained from Operon, contained two p53 response elements. The sequence of this oligonucleotide was the following: 5′-GAT CCC TGG TCA AGT TGG GAC ACG TCC GGC GTC GGC TGT CGG AGG AGC TAA GTC CTG ACA TGT CTC CG-3′. The SCS synthetic oligonucleotide used in this study, also obtained from Operon, contained one consensus p53 binding site. The sequence of this oligonucleotide was the following: 5′-TCG AGC CGG GCA TGT CCG GGC ATG TCC GGG CAT GTC-3′. The mutant oligonucleotide used was derived from the RGC sequence and was the following: 5′-TCGAGTTTAATGGACTTTAATGGCCTTTAATTC-3′. Labeling of the oligonucleotides was performed with the large fragment of DNA polymerase and ^{32}P-dCTP. Reaction mixtures for EMSA experiments (30 μl) were carried out with 0.1 pmoles of oligonucleotide, 0.5 μg salmon sperm DNA, 1 mM DTT, 1 mM EDTA pH 8.0, 100 mM KCl, 20 mM HEPES pH 7.8 and 10% Glycerol. In addition to 2 μg of 10-1 or 3-4 nuclear protein extract, 1 μg of PAb 421 was added to the designated reactions. In the reactions with purified p53 150 ng of protein was added. All samples were incubated at room temperature for 30 min. The protein–DNA complexes were resolved on a 4% acrylamide gel which was pre-run at 100 V at 4°C for 2 h prior to loading and samples were then electrophoresed at 200 V for 3 h.

Plasmid DNase I footprinting

Reaction mixtures for plasmid footprinting (50 μl) were carried out with 25 ng of the genomic sublcone digested with *Xho*I and *Apa*I in the presence of 1 μg of BSA, in 2 mM Spermidine, 0.9 mM DTT, 2 mM MgCl2, 0.1 mM EDTA pH 8.0, 25 mM KCl, 20 mM HEPES pH 7.5 and 10% Glycerol. Increasing amounts of p53 were added as indicated in the figure legend and the reaction mixture was incubated on ice for 30 min followed by digestion with 2 ng of DNase I (Worthington; 2932 units/mg) on ice for 1 min followed by the addition of 50 μl of DNase I stop buffer (2M ammonium acetate, 100 mM EDTA, 0.2% SDS, 100 μg/ml sheared salmon sperm DNA. The DNA was phenol and chloroform extracted followed by ethanol precipitation. The pellet was resuspended in 15 μl of dH_2O followed by primer extension with ^{32}P-labeled oligo #3 (5′-GGAATCTGTGAGGTGCTTGCAGCA-3′). 1.5 μl

of ^{32}P-oligo #3 (1 pmol/μl) was added to the reaction along with 0.5 μl of 10 × Taq buffer, 3 μl of 2.5 mM dNTPs and 0.5 μl Taq DNA polymerase. Seven PCR cycles of denaturation at 94°C for 1 min, annealing at 68°C for 2 min and extension at 74°C for 3 min were carried out and then 30 μl stop mixture (24 μl TE; 1 μl of 5 mg/ml tRNA; 5 μl of 3M NaOAc) was added, followed by chloroform extraction and ethanol precipitation. The pellet was resuspended in 12 μl of sequencing loading buffer and the sample was electrophoresed on a 6% denaturing polyacrylamide gel.

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2. Xiao, G., White, D. and Bargonetti, J. **p53 Binds to a Constitutively Nucleosome Free Region of the mdm2 Gene.** Oncogene, 16, 1171-1181, 1998.

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PERSONNEL:

Jill Bargonetti's release time salary